OBJECTIVES OF COAL BIOPROCESSING AND APPROACHES

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INTRODUCTION

There has been a great deal of excitement about various practical applications of biotechnology, including the production of chemicals, fuels, foods, and drugs; waste treatment; clinical and chemical analyses; toxicological assays; and uses in medicine. In fact, in the last four years there have been four issues of *Science* devoted to this "revolution" in biology. The two most recent were entitled "Biological Frontiers" (1) and "Biotechnology" (2).

Microbe-catalyzed processes constitute industrial microbiology, which today is a diversified multi-billion dollar industry (3,4). The applications of living microbial, plant and animal cells for the production of useful compounds has been extensive. The microbial conversions of lignocellulosic material, the biological precursor to coal, have also been studied in detail.

Studies in coal bioprocessing, however, is just getting underway and still in its infancy. Conceptually coal bioprocessing can be categorized into two areas: 1) coal cleaning--removal of undesirable components such as sulfur, nitrogen, trace metals; and 2) coal conversion--microbial liquefaction, microbial gasification, microbial pretreatment, methane production. The ability of any microbe or microbial consortia to break down a complex structure depends on the types of chemical bonds and the three dimensional environment around the bonds. Therefore, intertwined with coal bioprocessing is an understanding of the coal macromolecular structure and biochemical mechanisms by which microbes/enzymes break bonds. These relationships are depicted in Figure 1.

The objectives of microbial coal cleaning are clear. Removal of sulfur, nitrogen and trace metals by mild microbial processes for the production of clean coal is the overall goal. However, in the area of microbial coal conversion it seems that the objectives are not very clear. For example, in the case of microbial lignin degradation the objective is very clear; that is the complete degradation of the lignin. Obviously, one does not want to completely degrade the coal, but convert the coal into a more usable form by the application of microbial transformations.

Thus, the overall objective in any coal conversion scheme, whether microbial or chemical, is to produce a better fuel form, be it liquid fuel (transportation fuels), solid fuel, or even a new fuel form for direct utilization. This essentially entails depolymerizing of the coal macromolecule, removal of oxygen, increase in H/C ratio. This concept is depicted in Figure 2. It must be borne in mind that unlike in the lignin degradation, the coal can not just be subjected to a non-specific degradation, but must undergo microbial transformations resulting in a depolymerized, deoxygenated, denitrated product which would result in a better fuel.

The area of microbial conversion of coal is receiving a lot of attention these days. Cohen and Gabriel (5) reported that fungi could grow directly on and metabolize naturally occurring coal. Scott (6), Wilson (7), Ward (8), and Faison (9) have also reported degradation or solubilization of lignites by various fungal strains including the solubilization of a Wyodak subbituminous coal (6). However, little is known about the products of the degradation or solubilization. As
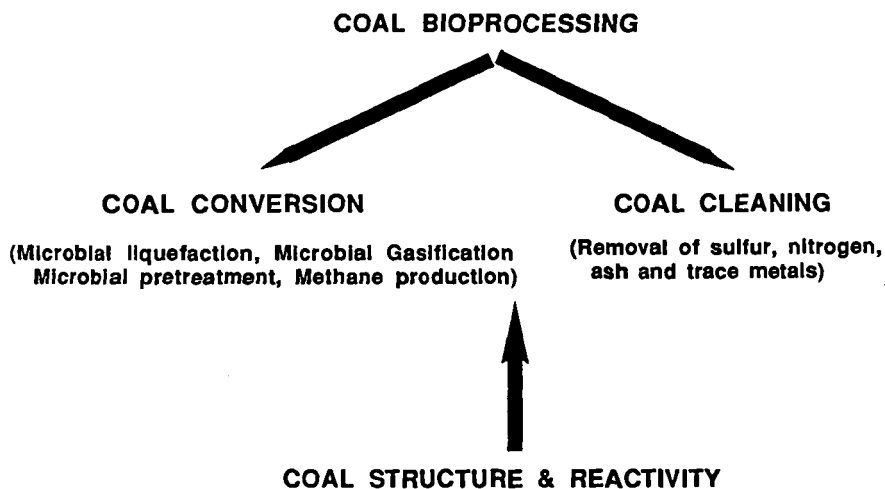
COAL BIOPROCESSING

COAL CONVERSION

(Microbial liquefaction, Microbial Gasification
Microbial pretreatment, Methane production)

COAL CLEANING

(Removal of sulfur, nitrogen,
ash and trace metals)

COAL STRUCTURE & REACTIVITY

Figure 1. Coal bioprocessing areas

NEW FUEL FORM ············▶ DIRECT UTILIZATION

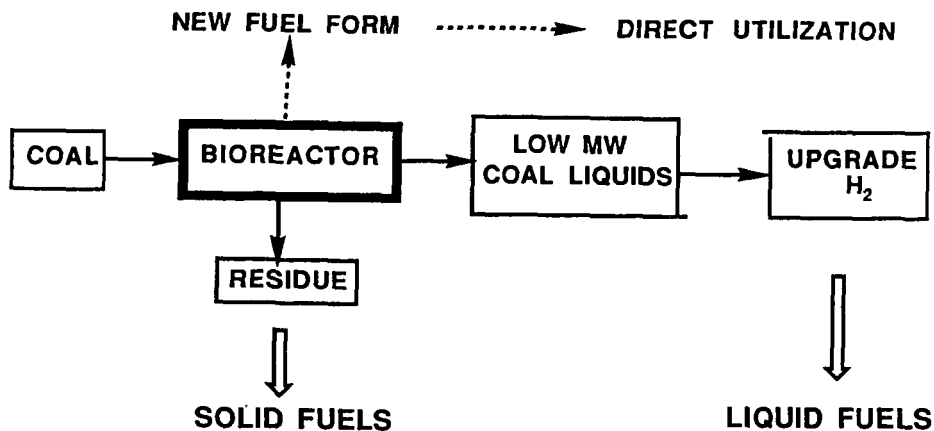| COAL | → | BIOREACTOR | → | LOW MW COAL LIQUIDS | → | UPGRADE H₂ |

RESIDUE

SOLID FUELS

LIQUID FUELS

Figure 2. Coal bioprocessing schematics

indicated earlier, the overall objective in any coal conversion scheme whether microbial or chemical is to produce a better fuel form.

Towards achieving this goal, there are two basic approaches to microbial processing of coal. The first approach involves the oxidative solubilization or depolymerization of the coal macromolecule. It involves using enzyme systems or microorganisms to hydroxylate aromatic rings followed by ring fission to a carboxylic acid. Since all of this involves introduction of oxygen into the coal, the question of whether coal is being converted to a less desirable fuel form (converting coal into biomass?) becomes an important issue. On the other hand, if the oxygenated material is more receptive to deoxygenation and/or upgrading than the starting coal, or has other uses, then this type of microbial conversion makes sense.

A second approach to microbial processing of coal, which, according to me promises to be much more rewarding and exciting. The approach envisions using facultative *anaerobic bacteria* to reductively depolymerize/solubilize coal, i.e., reduction of aromatic rings and reductive cleavages to product a hydrogenated product. In other words, hydrogenation of coal via an anaerobic microbial process resulting in a more desirable fuel form rather than oxidation via an aerobic microbial process which results in a less desirable fuel form. Indeed, coal scientists throughout the ages have been trying to do exactly this; i.e., inexpensive approach to product a depolymerized, hydrogen-rich coal fuel.

MICROBIAL COAL TRANSFORMATION IN AEROBIC SYSTEMS
(Oxidative Solubilization/Depolymerization)

All of the microbial conversions (degradation or solubilization) reported so far in literature involves aerobic systems. The mechanism operating under these conditions is oxidative, depolymerization that is hydroxylation of the aromatic ring followed by ring scission. Indeed, it has been shown that biodegradation/oxidation of aromatics are initiated by a series of enzymes known collectively as the oxygenases. The oxygenases can be further sub-divided into dioxygenases and monoxygenases. Molecular oxygen is essential for them to function since it is incorporated into the end product. The reaction pathway is shown in Figure 3 (10) with ring cleavage occurring at the bond between the hydroxyls (orthocleavage) or the bond adjacent to the hydroxyl. Two- (11,12) and three-ring aromatics (13-15) can also be degraded although free ortho ring positions must be available (Figure 4). Low-ranked coals have a number of hydroxy and dihydroxy aromatic structures, as well as carboxy groups. *Pseudomonas* species are capable of carrying out oxidation ring cleavage reactions (16-20) (Figure 5), or aromatic rings containing these functional groups. Therefore, the *Pseudomonas* species should be capable of oxidizing low-rank coals. It would be interesting to compare the products obtained from the microbial oxidative cleavage reactions with that obtained from the ruthenium tetraoxide catalyzed oxidation of the coal. This is because the mechanism of the ruthenium tetraoxide oxidations involves hydroxylation of the aromatic rings followed by ring cleavage which is similar to the mechanism of microbial oxidative ring cleavages.

Again, the question that pops up is whether such microbial processing approaches for depolymerization/solubilization of the coal makes sense. The reason being that we have introduced a number of oxygen functional groups during the process, which could result in less desirable fuel qualities.

Lignin Biodegradation

Lignin is considered as the precursor to coal and some young lignites may contain 35%-70% of lignin-like compounds. It is, therefore, not surprising that lignin degrading microorganisms and enzymes are being employed for microbial coal conversion. For example *Phanerochaete chrysosporium* and *Polyporous versicolor* have been used in microbial coal conversions.
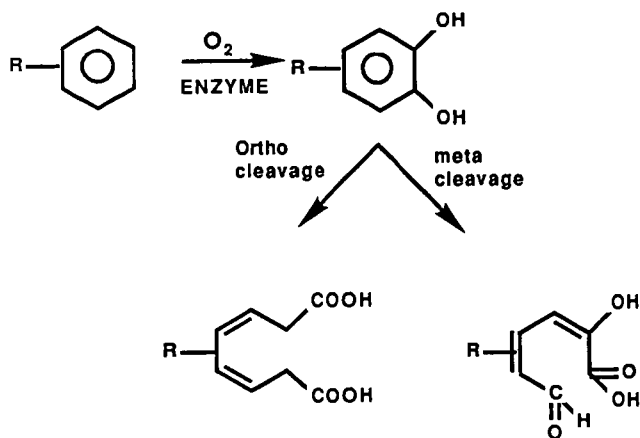
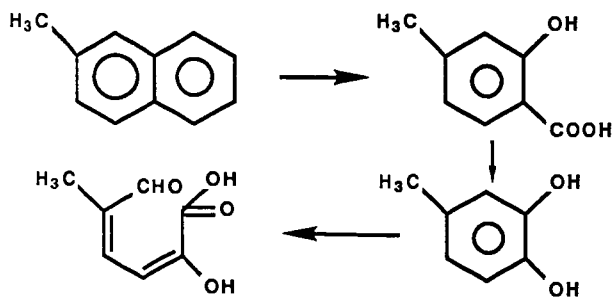Figure 3. Mechanism of aromatic degradation
by oxygenases

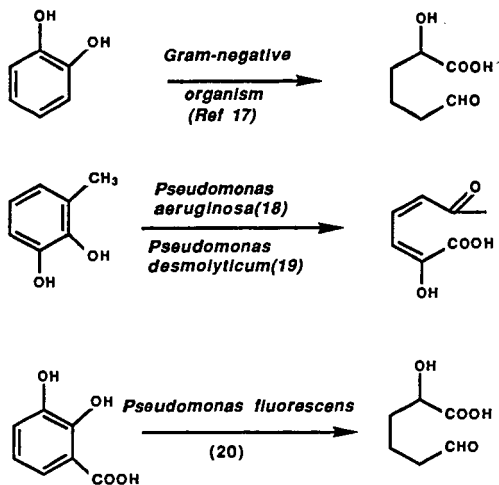Figure 4. Degradation of two ring compounds

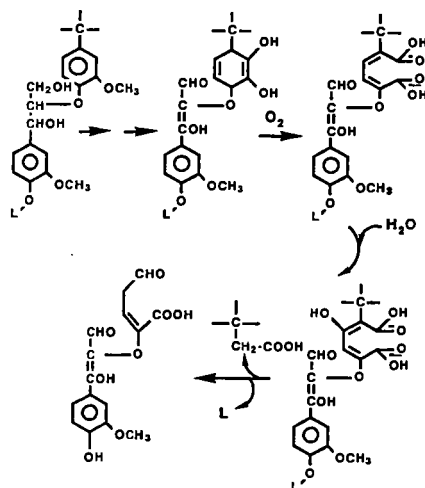**Figure 5. Oxdative ring opening reactions by *Pseudomonas***

Figure 6. Mechanism of lignin degradation

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These are "white rot" fungi and their mechanism of action for lignin degradation involving the enzymes ligninase and laccase have been extensively studied by Kirk and others (21-23). They report that the degradation proceeded by non-specific hydroxylation and ring opening just like the oxygenases functions and, not as previously believed, by beta-arylether cleavage initiated by a specific etherase enzyme (Figure 6).

ENZYMES VS WHOLE CELLS

The question also arises as to whether coal bioprocessing would be better achieved with growing cells or isolated enzymes. Multistep transformations such as the synthesis of interferon or the production of ethanol from cellulose involves a number of different enzymes acting sequentially and regeneration of co-factors is required. Therefore, it is clearly advantageous to use whole cells. For one step or two step transformations however, enzymes are probably superior because their use is free of drawbacks such as competing side reactions, sterility problems, and the cell lysis often associated with fermentations. Enzyme systems like the ligninases, oxygenase could promote selective transformations. There also exists the capability of overproducing the coal processing enzymes using recombinant DNA or genetic engineering techniques. In fact, the ligninase enzyme has been cloned in *Escherichia coli*. It therefore seems to me that the use of isolated enzymes for coal bioprocessing is advantageous and should be pursued.

In our laboratory, we are evaluating enzymes present in various microorganisms to carry out decarboxylation, hydrogenation and non-oxidative depolymerization of the coal. Preliminary results have shown that microorganisms such as *Bacillus megaterium* containing decarboxylase(s) can remove CO_2 from *model compound* vanallic acid and coal. The resulting coal has a higher H/C ratio. Low rank coals have a substantial number of oxygen tied up as carboxyls.

ANAEROBIC BIOCONVERSION OF COAL (Reductive Depolymerization/Solubilization)

A more exciting and potentially rewarding field is the area of coal bioprocessing under anaerobic conditions. To the best of our knowledge, almost no work has been done in this area. This approach envisions the use of facultative anaerobic bacteria to reduce the aromatic ring systems in the coal and produce a depolymerized hydrogenated product. In other words, instead of adding oxygen via an aerobic process and producing a less desirable fuel, we can add hydrogen to the coal using an anaerobic process and produce a richer fuel.

Anaerobic Aromatic Ring Metabolism

Under anaerobic conditions, aromatics are degraded by either hydration or hydrogenation followed by non-oxidative ring fission. In all cases investigated the microorganisms initially reduce the ring structure (24). Tarvin and Buswell (25) reported the complete utilization of benzoate, phenylacetate, phenyl propionate and cinnamate by anaerobes. Healy and Young (26) found that 11 simple lignin derivatives were biodegraded to methane and carbon dioxide under strict anaerobic conditions. Anaerobic degradation of two and three ring lignin fragments has been proposed to occur via an overlap of the ferulate and benzoate degradative pathways (27).

Cleavage of the benzene nucleus anaerobically occurs by at least three different reaction schemes as shown in Figure 7.

A. Photometabolism; ex *Rhodopseudomonas palustris*..

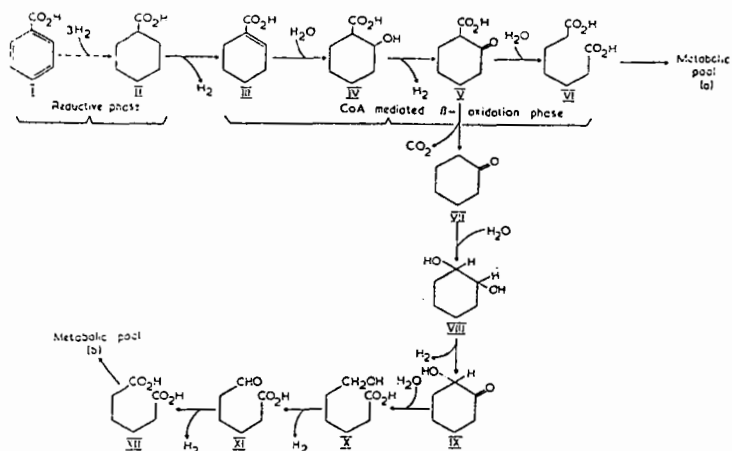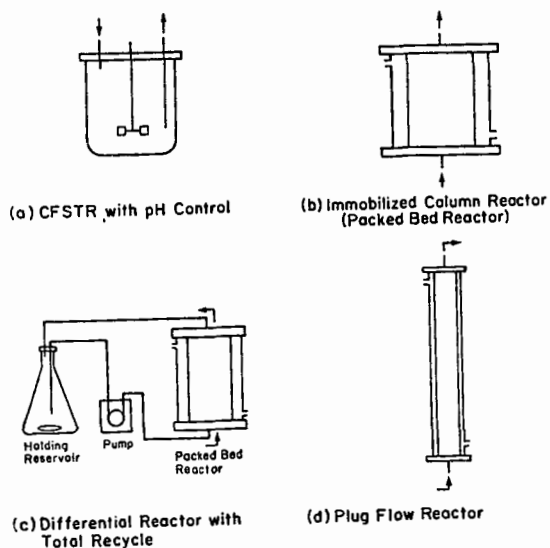Figure 7.   Anaerobic aromatic - ring metabolism

(a) CFSTR with pH Control

(b) Immobilized Column Reactor
(Packed Bed Reactor)

(c) Differential Reactor with
Total Recycle

(d) Plug Flow Reactor

Figure 8.   Bioreactor configurations.

B. Nitrate respiration; ex *Moraxella sp.*

C Methanogenic fermentation; ex occurs via a consortium of gram negative organisms and various methane bacteria.

Several species of the purple non-sulfur bacteria, the *Rhodospirillaceae*, are able to use aromatic compounds as sole carbon sources by photometabolism. When cell suspensions of *R. palustris were* incubated with (^{14}C) benzoate the seven carbon atoms of benzoate remained together until after the ring cleavage stage.   These results suggested a series of reactions involving the reduction of benzoate (or a derivative) to a cyclohexanecarboxylate moiety followed by a coenzyme-A mediated beta-oxidation sequence.   Because the reduced acid is acyclic, breakage of the bond in the 1,2 position occurs to yield pimelate (or its equivalent). This new reductive pathway is illustrated in Figure 7b.   Evans (28) has recently demonstrated the occurrence of these reactions using subcellular fractions from *R. palustris* grown photosynthetically on benzoate with the following results: (a) a washed chromatophore or benzyl-Co A under illuminated, anaerobic conditions; (b) a cell-free extract in the presence of CoA, ATP, NAD$^+$ and Mg^{2+} ions converted cyclohex-1-enecarboxylate to pimelate in anaerobic or aerobic conditions in the light or dark.

These results provide strong evidence for the presence of a light-dependent membrane bound proton-translocating redox system in these chromatophores; the low potential reductant may be a ferrodoxin, which also plays a part in photosynthetic electron transport.   In addition, they confirm the presence of the appropriate beta-oxidation suite of enzymes in these cells responsible for the subsequent series of reactions resulting in ring-cleavage.

This is an exciting approach to the bioconversion of coal.   One can take advantage of the capability of phototrophic bacteria for reductive bioconversion of aromatics to carry out coal bioconversions.

MICROBIAL COAL CLEANING

Unlike microbial conversions, the microbial cleaning objectives are much better defined. Removal of sulfur primarily trace metals, chlorine and nitrogen by microorganisms is the focus of attention.   Microbial desulfurization, especially, pyrite removal has received a lot of attention, and a number of papers by different research groups have been published on the subject (29,30).   *Thiobacillus* is used for pyritic sulfur removal and a host of literature is available on the subject.   The question arises whether it is cost-effective to use *Thiobacillus* or other microorganisms for pyritic sulfur removal because it takes weeks and even months for the reaction to go to completion.   A more viable option in which Atlantic Research Corp. and Illinois Geological Survey, are working in the use of microorganism to surface modify the pyrite is coal and make it more hydrophilic.   This type of conditioning takes only three to four hours and a conventional flotation process can now eliminate almost all of the pyritic sulfur from this microbially pretreated coal.   Another approach would be to let loose the microorganism in a coal storage pile and then come back later to have a sulfur-free coal available.   The idea is not as far fetched as one thinks.   Indeed, in Cuba, such an approach is used for the removal of lignin from cane sugar--called the Cubanine process. Lignin-degrading enzymes from the white rot fungus are put together with the biomass cane sugar material in a large chamber and after a month has elapsed the biomass is taken up, ground and made ready for use.   The Cubans have found that this method is cost-effective in removing lignin and saves them a lot of energy which they would, otherwise, have to expend to remove the lignin.

BIOPROCESS ENGINEERING COMPONENT

Finally, one has to recognize that in order for these microbial conversions to reach a commercial scale, bioprocess considerations are warranted. Bioreactor design, problems about nutrient recycle or other process considerations entails the fact that bioreactor engineering will have to become an integral component of any coal processing scheme. Figure 18 illustrates some of the possible bioreactor configurations that can be used.

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MICROBIAL DESULFURIZATION OF SOME EUROPEAN COALS: PROGRESS REPORT ON A JOINT ITALIAN PROJECT

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1. INTRODUCTION

The continuing fluctuation of the oil market together with the likelihood of a rise in the current relatively low prices, high-light the ever-present problem of alternative energy resources availability. This problem, of worldwide impact, is particularly pressing in those industrialized countries which, like Italy, can rely on only very limited domestic energy resources and, on account of political decisions, must refrain from resorting to nuclear power.

In the strive towards the exploitation of all available domestic energy resources, The Italian state-owned energy supply company, ENI-AGIP, has reopened the Seruci mine in the Sulcis subbituminous coal basin (Island of Sardinia). The mine is expected to reach full production of two million tonnes per year of marketable coal by 1992. The entire production is intended for the electrical power stations operating in Sardinia. Sulcis coal has the characteristics shown in Table 1: its major drawback is its high sulfur content. Current legislation on industrial air pollution in Italy restricts peak concentrations of SO_2 in power station emissions to 657 micro-grams per cubic meter in 30 minutes and 263 micrograms per cubic meter over 24 hours. Even more stringent are the limits suggested by the European Economic Community (EEC). These standards all impose severe limitations on the sulfur contents of coals since they vir-tually equate to maximum permissible sulfur contents of about 0.8%.

On the other hand, the sulfur contents of world coal reserves vary from 0.38 to 10%, and that of mineable reserves from 0.38 to 5.32%, which means that a considerable portion of the coal mined through-out the world cannot be directly used for combustion without some preliminary measures being taken to keep, in one way or another, emissions within the prescribed limits. Table 2 shows the pollution potential, in terms of grams of SO_2 per MJ of typical coals extrac-ted from European and American mines.

Although most coal mined in the EEC is presently extracted from those parts of the respective basins containing low-sulfur coals, (i.e. coals containing less than 1% total sulfur), the economic importance of the possibility of mining high-sulfur coals too has been given full recognition by the EEC. Thus, in 1986, the EEC launched an R & D programme with the objective of assessing the possibilities offered by biohydrometallurgy in microbial coal de-sulfurization. This programme is now well under way and four coun-tries are participating therein: Holland (Biotechnology Delft), Italy (University of Cagliari), the United Kingdom (Warren Springs Laboratories, Stevenage) and West Germany (Bergbau-Forschung GmbH).

In Italy the programme is also partly supported by the Italian company ENICHEM-ANIC, which, in 1986, signed a research contract with the Mining and Mineral Dressing Department (MMDD) of Cagliari University. Part of this research project has been entrusted to researchers of the Microbiology Institute of the 'La Sapienza' University (MILSU) in Rome, which is thus closely cooperating with the MMDD of Cagliari University.

The present paper is intended as a progress report on the problems encountered and the results achieved by the two above-mentioned Italian universities.

2. SULFUR COMPOUNDS AND THEIR IDENTIFICATION

Coal contains sulfur in four forms: metallic sulfides, with a pre-dominance of iron sulfides ('pyritic sulfur'), atoms covalently bound in the organic sulfur compounds contained in the coal matrix ('organic sulfur'), iron, calcium sulfates and elemental sulfur. Pyritic and organic sulfur are the most common: elemental sulfur does not usually assay more than 0.2% and sulfates more than 0.1%. Moreover, being soluble in water, the sulfates can be readily re-moved during coal washing. Whilst pyritic sulfur can be very accu-rately identified with several procedures (reflected light micro-scopy, ordinary chemical analysis), much less is known about the nature of the organosulfur compounds forming the coal matrix and it is still the object of intensive research and debate. As a mat-ter of fact, there are no reliable, direct and single methods for quantitatively determining the organosulfur compounds in coal and even their qualitative determination is considered by some workers to suffer from too many uncertainties (1). It has been suggested that organic sulfur is present in coal in at least five main forms, which correspond to the functional groups containing aliphatic or aromatic thiols, aliphatic, aromatic or mixed sulfides and/or disulfide heterocyclic compounds containing the thiophenic ring and the gamma-thiopyrone system (2),(3).

Although no evidence exists of the direct determination of these compounds, the well known diagram of Figure 1 has been proposed (4): it represents the molecular structure of a typical bituminous coal where the sulfur atoms bound in the organic molecules are indicated with arrows. According to Meyer (2), the average pyritic sulfur content of the world's coal reserves ranges from 0.09 to 3.97% and the organic sulfur content from 0.29 to 2.04%. In some high-sulfur coals, like those investigated herein, these percentages can be higher with predominance of organic sulfur. Hence, coal depyritization, be it carried out microbially or by other means, can only produce coals complying with environmental specifications when organic sulfur is negligible. Otherwise, the organic sulfur must necessarily be removed.

For the time being organic sulfur removal can only be attempted by chemical processing (5): however, the costs involved are prohibitive and discourage commercial application. These considerations warrant the investigation of the possibilities of microbial organo-sulfur removal.

3. MATERIALS AND METHODS

3.1 The Investigated Coals

The programme entails the investigation of the amenability to micro-bial desulfurization of four coals: Sulcis coal, from the Sulcis coal basin in southwestern Sardinia, Monopol coal, from the Monopol coal mine in West Germany, Gardanne coal, from Provence in southern France and a Spanish coal from the Teruel mine. At the time of writing the Teruel coal had not yet been delivered to Cagliari's laboratory. Tables 1, 3 and 4 summarize the most salient character-istics of the above-mentioned coals.

3.1.1. Sulcis coal

On the grounds of 'rank' determinations based on the average re-flecting power of vitrinite, Sulcis coal can be considered a sub-bituminous coal according to the ASTM (USA) classification or a 'Glanzbraunkohle' according to the DIN (West Germany) classifica-tion. If the additional parameter represented by the upper thermal power - determined on pure coal and with its intrinsic moisture, in compliance with the latest EEC guidelines - is considered, then Sulcis coal can be placed among the 'average rank' coals, near to the limit of 'lower rank' coals. Its maceral composition is characterized by an abundance of the 'vitrinite' group, which in the I Seam ranges from 70 to 75%. After the vitrinite group, the maceral group exinite-liptinite is the most represented, with cuti-nite predominating: in the I Seam, the exinite-liptinite associa-

tion amounts to 12 to 24%. The maceral group inertinite ranges from 2 to 7%. The main associated mineral phases are dolomite, calcite, pyrite, marcassite, quartz, aragonite and clay.

Pyrite, which is present throughout the Sulcis coal, is syngenetic and finely dispersed within the coal matrix (Fig. 2), where it occurs as individual crystals from submicron to micron size, along with less frequent framboids no larger than 40-50 micrometers. This very fine intergrowth makes pyrite removal with classical physical mineral dressing methods impossible. Pyritic sulfur is always accompanied by higher proportions of organic sulfur.

3.1.2 Gardanne coal

The 'rank' and technological determinations indicate that this coal belongs to the class of 'subbituminous' coals (ASTM). The Gardanne coal is predominantly composed of vitrinite (up to 65%) and, to a lesser extent, of inertinite (inertodetrinite, semifusinite, fusinite) and exinite-liptinite (cutinite, resinite, sporinite). It is characterized by abundant occurrence of pyrite and carbonate phases (dolomite and calcite) accompanied by quartz and clay.

Pyrite, basically syngenetic in nature, is finely dispersed within the coal matrix in the form of individual crystals (sometimes submicronic in size) and of framboidal clusters ranging in size from 5 to 40 micrometers (Figure 3).

Organic sulfur is always present in high percentages: investigations with the electron microprobe revealed that vitrinite contains more organic sulfur than other macerals.

3.1.3 Monopol coal

This is a bituminous coal containing, in decreasing order, vitrinite, exinite and inertinite. Vitrinite occurs as bands of variable thickness, alternated with exinitic and inertinitic bands. The exinite group is predominantly composed of sporinite, cutinite and resinite, whereas the major components of inertinite are fusinite and semifusinite.

Pyrite occurs in Monopol coal in both the syngenetic and epigenetic form. The syngenetic pyrite appears as individual framboids ranging in size from a few micrometers up to 40 micrometers, with a predominance of the 20 micrometer size, and less frequently as groups of framboids. Epigenetic pyrite is present in the form of fissure fillings (Fig. 4) and is frequently accompanied by marcassite. The veins of epigenetic pyrite range in thickness from 20 to 300 micrometers, with 100 micrometers prevailing.

Pyrite is also present in cellular cavities of semifusinite and fusinite structures. Of the carbonates, calcite, and to a lesser extent siderite, can be mentioned. Quartz and clay were also detected.

3.2 The Microbial Strains

3.2.1 Pyritic sulfur removal

For pyrite removal a Thiobacillus ferrooxidans active mixed culture was used, isolated from acid drippings of the Fenice Capanne Mine (Tuscany, central Italy) (6) routinely maintained in Cagliari's laboratory in Silvermann and Lundgren 9K medium.

3.2.2 Organosulfur removal

To the best of the authors' knowledge, only one strain of Pseudomonas sp., denoted CB1 strain and listed as ATCC No. 39381 , obtained by chemical mutagenesis of a wild strain, is claimed to be so effective in removing some organosulfur compounds from coal (7), as to encourage its testing with a view to commercial application. Sulfolobus acidocaldarius is also claimed to be capable of removing some organic sulfur from coal, although it does not perform as well (8). The CB1 strain and Pseudomonas putida PAW 340 were therefore also investigated, at least for comparative purposes.

A campaign of microbial strains collection was undertaken with the aim of isolating microorganisms present in a model mining environment. The following procedure was adopted for isolating microorganisms from the Seruci mine: a 20 dm^3 Mariotte carboy, containing the suitable culture medium, was placed on a platform supported by a steel arch of a gallery located at 250 meters below sea level, where the temperature was 35°C. The medium was allowed to spread and percolate over the coal exposed on the gallery wall and was finally collected in another carboy placed on the gallery floor (Fig. 5). Repeated samplings, carried out with complete and minimal media as well as minimal media supplemented with selective agents, led to the isolation of the following strains:
- from complete medium Bacillus sp., Enterobacter, sp. and Micrococcus sp.;
- from minimal medium, and minimal medium supplemented with dibenzothiophene (DBT) as sole carbon source, several Gram-positive and Gram-negative bacteria, with a prevalence of strains belonging to Bacillus and Pseudomonas genera.

The isolated microorganisms were serially subcultured at 30°C and 26.17 rad.s^{-1} in 300 cm^3 flasks containing 50 cm^3 of basal salt

solution M9 (9), supplemented with 5% powdered coal or a minimal medium supplemented with DBT).

3.2.3 Leaching techniques

All the sulfur removal tests were carried out in 300 cm^3 Erlenmeyer flasks containing either organosulfur compounds presumably present in the investigated coals (e.g. DBT) or coal along with the suitable culture medium. They were incubated on giratory shakers operated at the speeds and temperatures specified in the descriptions of the individual runs.

3.2.4 Reagents

All reagents used in the tests were analytical grade. The culture media were always made up with distilled water and, for laboratory testing, were always sterilized.

3.2.5 Analytical methods

Total sulfur was determined by means of the SC132 Instrument manufactured by LECO (St. Joseph, MI, USA). Pyritic sulfur was determined by gravimetric analysis, whereas iron and other elements were analysed by complexometric titration. Organic sulfur was determined as the difference between total and pyritic plus sulfate sulfur. The analyses of the residual DBT after microbial attack were carried out with an original method developed by the Industrial Chemistry Department of Bologna University (10).

Hydrogen ion concentrations were determined by means of potentiometric pH-meters of various makes (Beckmann, Orion and Hanna) and redox potential measured with a Mod. HI 8418 Hanna electronic potentiometer with Pt combined electrode.

3.2.6 Microbial growth measurements

Cell concentrations were obtained by the dilution plating method and by counting colony-forming units (CFU) per cm^3.

4. RESULTS

4.1 Pyritic sulfur removal

Tests were carried out on Sulcis, Monopol and Gardanne coals, the coal from Teruel (Spain) being unavailable.

4.1.1 Sulcis coal

All tests were performed in 300 cm^3 Erlenmeyer flasks with baffled bottom, containing suspensions of coal powder in 9K medium. The shaker speed was adjusted to 26.3 $rad.s^{-1}$ and temperature maintained at 28±1°C. CO_2 was added to the shaker atmosphere. Owing to the very fine intergrowth of pyrite within the coal matrix and in spite of the relatively high porosity of the coal, a satisfactory access of the solutions and/or microbes to the pyrite crystals can only be achieved provided that most of them are at least partially exposed. Hence grinding to -40 micrometers is required to achieve 90% pyritic sulfur removal in a leaching time ranging from 9 to 12 days. These pyrite removals were achieved for solids-to-liquid ratios (weight of coal divided by weight of liquid medium) ranging from 1:100 to 16:100. Poorer results were obtained for solids-to-liquid ratios higher than 20:100. The initial pH of the medium was adjusted to between 2.20 and 2.25, and pulp acidity had to be stabilized prior to inoculation with about 45 Kg of concentrated sulfuric acid per Mg of coal. The final pH of the leach liquor was usually 1.8. The redox potential of the medium steadily increased from about 370 mV to more than 650 mV.

4.1.2 Monopol coal

Several runs were carried out in the same conditions as those adopted for Sulcis coal, but the results were quite deceptive. Some runs lasted up to 25 days, but no appreciable pyrite removal was observed. The pH of the leach liquor only dropped very slightly (from 2.20 to 2.00) and the redox potential of the solution never rose above 540 mV. Viability tests showed however that microorganisms remained viable well after conclusion of the tests.

Factorial variance analyses are presently under way, with potassium, phosphorus and ammonium sulfate concentrations of the medium as variables.

4.1.3 Gardanne coal

A first series of tests was carried out in the same experimental conditions as those adopted for Sulcis coal. Pyrite removal appears to be of the same order of magnitude, but always lower, as that achieved with Sulcis coal: the solubilization rate is constantly about 20% slower; Preliminary tests carried out on this coal varying phosphorus, potassium and ammonium sulfate concentrations in the medium, seem to indicate that a suitable composition of the medium might considerably improve the effectiveness of microbial attack.

4.2 Organic Sulfur Removal

All the isolated strains were analysed in order to assess their
ability to grow in basal salt solution supplemented with powdered
coal (5% w/v) or a selective agent (1 per thousand DBT w/v). The
data obtained show that all strains can grow on basal salt solution
supplemented with coal. On the other hand, only the strains pre-
selected in the mine using basal salt solution plus DBT exhibited
significant growth when cultivated in the presence of this select-
ive agent.

As for coal-degrading activity, removal of between 10 and 18% orga-
nic sulfur was observed. Under these experimental conditions, no
degrading activity was detected for the CB1 strain.

It should be noted that for sulfur contents of the order of magni-
tude of those of the examined coals (from 1.5 to 6.0%), a certain
unpredictable variability was observed in the data provided by the
described analytical techniques. This fact stresses the need for
analytical methods of adequate sensitivity to detect fraction per-
cent variations in sulfur contents, presently lacking.

The nutritional characteristics of the above-mentioned microorgan-
isms are shown in Table 5.

5. REMARKS AND CONCLUSIONS

The occurrence in several EEC countries of sizeable reserves or
high-sulfur coals containing large proportions of organic sulfur
as well as the economic potential of the high-sulfur coal zones ex-
hibiting similar characteristics in several coal basins, provide
an impetus for needed research aimed at removing both types of sul-
fur.

Microbial removal of pyritic sulfur is, in principle, a well-defi-
ned process, and the effectiveness of Thiobacillus ferrooxidans in
dissolving the accessible pyrite from the coal matrix is well docu-
mented (11),(12),(13). However, the present research has emphasized
the major role played by the chemical composition of the run-of-
mine coal. The presence of phosphorus, potassium and nitrogen com-
pounds may, in effect, alter the composition of the nutrient medium
to such an extent as to diminish the effectiveness of microbial ac-
tion, probably due to substrate inhibition. It is therefore advis-
able to always design the composition of the basal salts medium ac-
cording to the type of processed coal.

Exposure - not necessarily liberation - of the pyrite component is
an essential condition for its microbial removal. Hence, petro-

graphic and porosity analyses of the coal should always provide the
information required to avoid excess of expensive grinding. As
shown by the diagrammatic sketch of Fig. 6, porosity effectively
contributes to the accessibility of solutions and/or microorganisms
to pyrite crystals not exposed on the coal grain surface.

Information on microbial organosulfur removal is very scanty. Among
the strains claimed in the literature (7),(8) to be effective to
a certain extent are Sulfolobus acidocaldarius and Pseudomonas
strain CB1 obtained by chemically induced mutation, although little
has been published on the organosulfur removal ability from coal .
The tests carried out to date in the present investigation do not
seem to support the effectiveness claimed for strain CB1 in remov-
ing organic sulfur. On the other hand, the organic-sulfur degrada-
tion ability exhibited by the strains isolated from the mine en-
vironment, appears encouraging.

One hundred percent sulfur removal cannot be achieved inasmuch as
the sulfur atoms are dispersed throughout the coal matrix and
therefore only those exposed on the coal surface (grain boundaries
or pore surfaces) can be removed. The larger the surface-to-volume
ratio, the more the sulfur is amenable to microbial attack. However,
the surface-to-volume ratio cannot be increased beyond a certain
value, owing to the high grinding costs and to the depreciation of
coal when its grain size is smaller than market specifications. For
the time being, the finest coal particles find commercial applica-
tion in the coal-water mixtures technique, with an average grain
size of 40 micrometers.

It is therefore imperative to exploit other means for increasing
the exposed surface. One possibility is preliminary depyritization
and dissolution of the carbonate phases present in coal (Fig. 7):
the surfaces of all the cavities left by pyrite and carbonates re-
moval contribute to coal matrix, and hence organic sulfur, exposure.
On these grounds, a tentative flowsheet for microbial coal desulfu-
rization is proposed in Fig. 8. This flowsheet should be considered,
at the present state of progress of the research, as a guideline
for the planning of laboratory and pilot plant testing.

It should finally be pointed out that the present inability of ana-
lytical chemistry to provide reliable analytical methods for quan-
titatively determining the organosulfur compounds forming the coal
matrix contributed to the difficulties encountered in evaluating
the organic sulfur removal by the above-mentioned microorganisms.

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Table 1 - Data of a typical Sulcis coal

Analysis of macerals %		Chemico-technological analysis		Reflective power of vitrinite %
Vitrinite	76.4	Ash	7.7 %	0.49
Exinite	15.8	Sulfur, total	5.9 %	
Inertinite	3.8	Pyritic sulfur	1.06%	
		Sulfate sulfur	0.8 %	
		Iron, total	1.4	
		CaO	20.5 % of ash	
		K_2O	n.d.	
		MgO	11.2 % of ash	
		P_2O_5	0.8 % of ash	
		Heating power (dry basis)	20.485 $kJ.kg^{-1}$	
		Porosity	31.46	

n.d. = not detected

Table 2 - Examples of average sulfur, ash and energy content of several coal samples

Country/State	County	Coal Seam	kJ/kg	% Ash	Total S %	g SO_2 per MJ
France		Gardanne	28,360	10.84	5.67	4.0
Italy	Sulcis	I Seam	27,900	10.81	6.46	4.6
Spain		Berga	15,900	41.0	4.75	6.0
		Utrillas	21,850	18.6	7.92	7.2
USA						
-Ohio	Belmont	Pittsburgh	28,600	15.1	6.19	4.3
	Coshocton	Middle Kittanning	28,360	14.1	6.39	4.5
	Muskingum	Lower Kittanning	28,800	11.5	4.85	3.4
-Pennsylvania	Indiana	Lower Kittanning	31,380	12.9	4.68	3.0
-Utah	Emery	Blind Canyon	31,850	5.6	0.58	0.3
-Virginia	Wise	Taggart	33,940	2.9	0.79	0.5
-W.Virginia	Preston	Upper Freeport	28,800	18.5	2.44	1.7
	Nicholas	Middle Kittanning	27,900	20.2	1.06	0.8
-Wyoming	Sweetwater	Rock Spring No.11	29,060	6.5	0.97	0.7

Table 3 - Chemico-technological characteristics of Monopol coal

Component	Weight %	Porosity %
Ash	28.5	4.5
Sulfur, total	1.97	
Pyritic sulfur	1.33	
Sulfate sulfur	0.21	
Iron, total	2.6	
Carbonates	0.4	% of ash
-K_2O	4.4	% of ash
-Na_2O	1.1	% of ash
-MgO	1.9	% of ash

Table 4 - Chemico-technological characteristics of Gardanne coal

Component	Weight %	Porosity %
Ash	7.5	29.09
Sulfur, total	5.1	
Pyritic sulfur	1.06	
Sulfate sulfur	0.21	
Iron, total	0.7	
Cao	20.0	% of ash
K_2O	0.3	% of ash
MgO	3.4	% of ash
P_2O_5	2.3	% of ash

Table 5 – Nutritional characteristics of microorganisms isolated in
          Seruci coal mine

Colony	Isolation Medium	Glucose 2%	M9 supplemented with NaBenzoate 1.5%	NaBenzoate 1.5%+DBT 1%	DBT 1%
1N	M9+glucose 2%	+	+	+	+
2P	M9+NaBenz. 1.5%	+	+	+	+
1Q	M9+NaBenz.+DBT	+	+	+	+
1R	M9+DBT 1%	+	+	+	+
1H	McConkey	+	+	+	+
1A	LB	–	–	+	–
1B	LB	+	+	+	–
1S	MMA+coal 5%	+	+	+	–
1L	M9+glucose 2%	+	–	+	–
1M	M9+glucose 2%	+/–	–	–	+
2A	LB	+	–	–	–

1 = strains isolated in mine by means of M9+DBT
2 = strains isolated in mine by means of M9

N.B. The strains capable of growing only in the presence of glucose
      as carbon source are not reported, except 2A, which has been
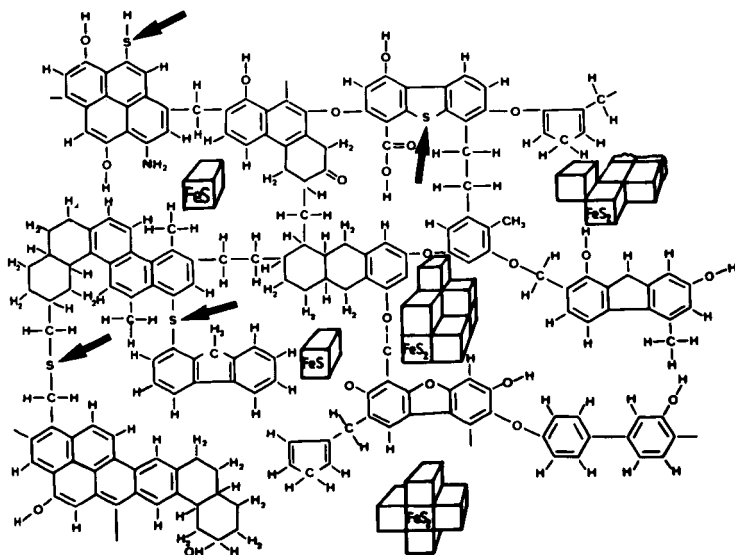   used as control.

Figure 1 – Molecular structure of a typical bituminous coal after Wendell
Wiser. The sulfur atoms bound in organic molecules are indica-
ted by arrows.(Modified from Wheelock ( )).
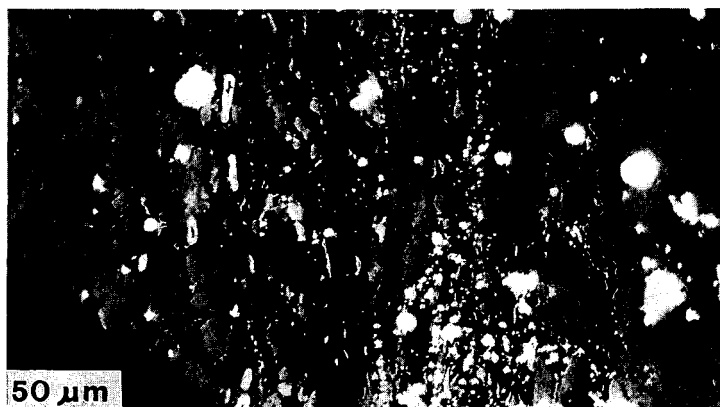

50 µm

Figure 2 – Pyrite in Sulcis coal: fine dispersions; refl.light, oil
immersion.

Figure 3 - Pyrite in Gardanne coal: framboids;   refl. light, oil
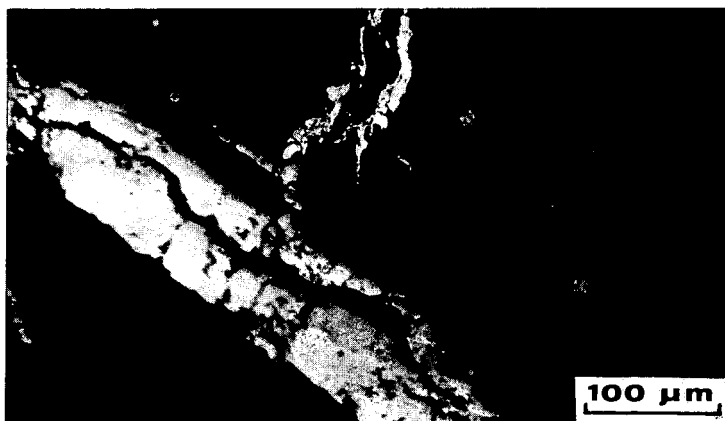   immersion.

Figure 4 - Pyrite in Monopol coal: epigenetic veins in cleats;
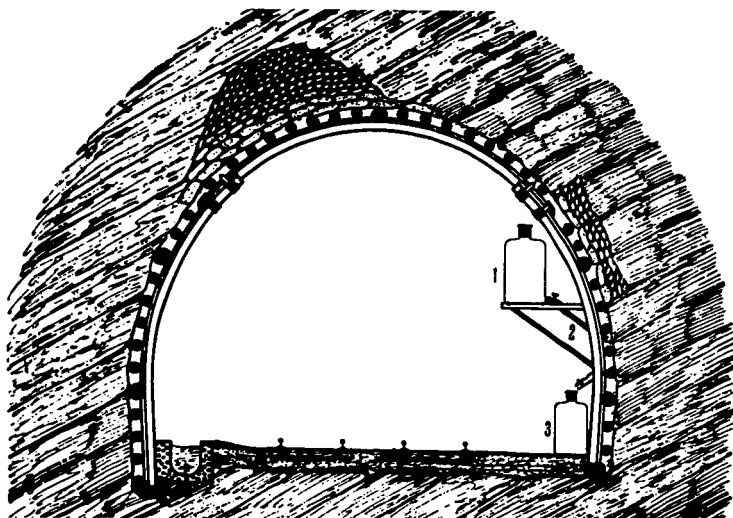   refl.light, oil immersion.

Figure 5 – Set-up for collecting microbes in the Seruci mine.
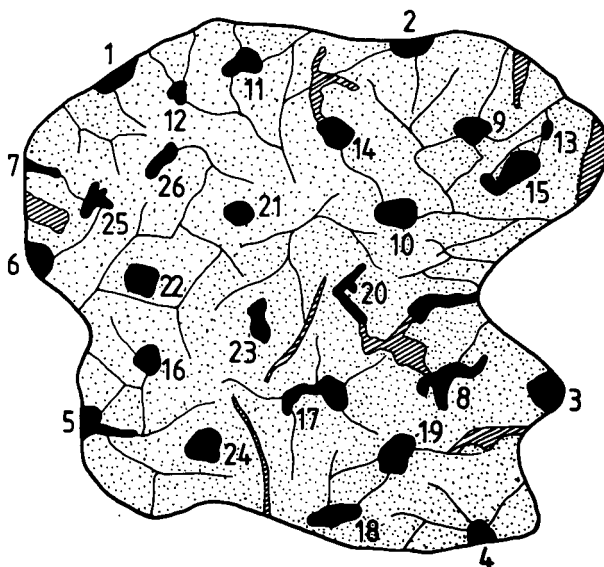1 = head tank;   2 = pipe;   3 = medium collecting carboy.

Figure 6 – Two-dimensional model of coal particle. Black grains are py-
rite; dashed grains are carbonates; white matrix is coal;
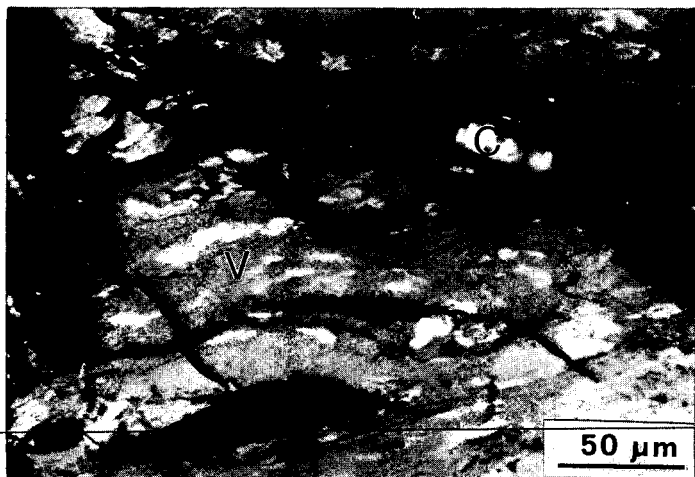lines are pores or cracks.

Figure 7 – Sulcis coal: carbonates (C) in fissures in vitrinite (V); refl.light, oil immersion.
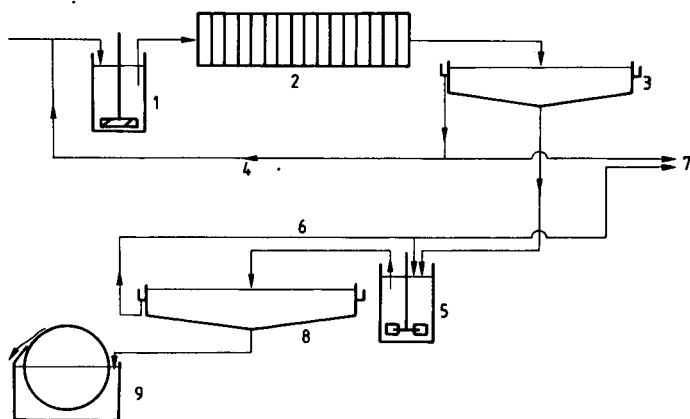

Figure 8 – Microbial coal desulfurization flowsheet. 1: backmix reactor; 2: plug flow reactor for pyrite removal; 3: thickener; 4: medium recycle; 5: backmix reactor for organic sulfur removal; 6: supernatant to waste; 7: tailings disposal; 8: thickener; 9: filter.

BIODEGRADATION OF COAL-RELATED MODEL COMPOUNDS

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INTRODUCTION

Since 1982 when Cohen and Gabriele (1) first reported that fungi could grow directly on and metabolize naturally occuring coal, biological conversion of low-rank coals by bacteria, fungi, or preparations of the enzymes they produce has been the subject of intensive research. Because these processes occur at ambient temperatures and pressures, they represent a potential savings in the processing of certain coals and lignites. Current technology for coal conversion requires both high temperatures and pressures which may result in the production of components that are more toxic than the original starting material.

Cohen and Gabriele (1) reported that fungi could metabolize leonardite, a naturally oxidized form of lignite coal. Wilson et al. (2) have shown that the leonardite-biodegraded product from *C. versicolor*, a white-rot fungus, was water soluble and contained no detectable polycyclic aromatic hydrocarbons (PAH) having three to six rings. In addition, the bioconverted material was inactive in the microbial histidine reversion assay for mutagenic activity. Linehan et al. (3) determined by gel permeation chromatography that the average molecular weight of the biodegraded product was 1800 daltons.

The details of the specific reactions of lignin biodegradation, and the biochemistry involved, have been primarily based on the use of low molecular weight compounds representing specific substructures rather than the complex, polymeric lignin material. As an example, Gold et al. (4) utilized ß-aryl ether model compounds to examine oxidation by lignin peroxidase from *P. chrysosporium*. We have studied the reactions of model compounds having coal-related functionalities (ester linkages, ether linkages, PAH) with the intact organism, cell-free filtrate, and cell-free enzyme of *C. versicolor* to

better understand the process of biosolubilization. Many of the degradation products have been identified by gas chromatography/mass spectrometry (GC/MS).

EXPERIMENTAL

Preparation of the Enzyme (5). The fungus was grown in a Chemap CF-20 fermentor at 25° C, and was supplied with 4 L of filtered air per minute at atmospheric pressure to 15 L of growth medium. The growth medium used was that published by Fahraeus and Reinhammar (6). A two-stage Rushton turbine (6 blade) agitator system was used at a speed of 400 rpm. The inoculum used for the fermentation was three 50 mL cultures of *C. versicolor* grown on Sabouraud-maltose broth (1) for 7 to 10 days at 25° C with no agitation. The fungi grown under these conditions form a mycelial mat. The mats were then transferred to a stoppered vessel containing 250 mL of distilled water and 50 mL of 3-mm glass beads. This vessel was shaken vigorously and the mycelial fragments were transferred to the fermentor. After three days growth in the fermentor, 0.3 mL of 2,5-xylidine (Aldrich Chemical Co., Milwaukee, Wi) was added to increase levels of extracellular laccase (5).

The extracellular fluid formed during the growth of *C. versicolor* was separated from the cell mass by filtration through several layers of cheesecloth. This fluid was termed the cell-free filtrate. The resulting fluid was then filtered through an ultrafiltration membrane with a molecular weight cutoff of 100,000 (Amicon H5P100-43). The higher molecular weight material in aqueous fluid was concentrated using an ultrafiltration membrane with a molecular weight cutoff of 10,000 (Amicon H5P10-43). The remaining fluid was further washed (diafiltered) with three 300-mL portions of water while being continually passed through the H5P10-43 membrane to maintain a constant volume of 1 L. The concentration and diafiltration steps caused precipitate formation which was removed by either filtration or centrifugation. The concentrated, extracellular fluid was then chromatographed on a 2 cm X 30 cm DEAE-cellulose (Amicon cellufine DEAE-AM) column which had been previously equilibrated to 0.01 M sodium phosphate, pH 7.0 buffer. This final fluid was termed the cell-free enzyme.

Model Compound Preparation. All of the model compounds were prepared in the same manner. Approximately 10 mg of each was dissolved in 10 mL of methanol. Suitable volumes of this stock

solution were added to the incubation system to make the desired concentration in water.

   C. versicolor was cultured on Kirk's minimal medium (10 mL) for ten days in 125-mL Erlenmeyer flasks.   One hundred microliters of benzylbenzoate and methoxybenzophenone were added to the culture.   Controls consisted of (a) cultures with the same volume of methanol added as with the model compounds and (b) benzyl benzoate and methoxybenzophenone were added to the sterile minimal medium.   Hyphal growth of fungal cultures was evident following addition of methanol indicating that the volumes of methanol added were not inhibitory.   The model compounds   and intact organism were incubated for 1 week.   The cultures were then filtered through 0.2 μ m filters and extracted with three equal volumes of chloroform.   The extracts were combined, concentrated to 1 mL, and analyzed by gas chromatography/flame ionization detection (GC/FID) and GC/MS.

   Dibenzothiophene, indole, and bibenzyl were incubated with *C. versicolor* for three weeks.   The samples were extracted and concentrated in the same manner as discussed above.   Controls, as discussed previously, were also obtained and analyzed.

   Benzylbenzoate, bibenzyl, phenylbenzoate, methoxybenzopheneone, and benzyl ether were added (100 μ L) to a mixture of 1 mL of cell-free filtrate and 1 mL of buffer solution (pH 5.2).   After one day, the solutions were extracted with three equal volumes of chloroform.   The extracts were combined and concentrated to 1 mL for subsequent GC/FID and GC/MS analysis. Controls were made with the model compounds and the buffer solution.   The controls were extracted, concentrated, and analyzed in the same manner.   Anthracene was incubated with the cell-free filtrate for three days.   The solution was then extracted, concentrated, and analyzed by GC/FID and GC/MS.

   Benzylbenzoate and methoxybenzophenone were introduced (100 μL) to the cell-free enzyme and a buffer solution at pH 5.2. After equilibration of the enzyme and buffer for one day, the solution was extracted, concentrated, and analyzed in the same manner as discussed previously.

   Extraction efficiencies were determined by GC analysis of a solution of the model compound in the medium that did not contain

enzyme. The peak areas were then compared with the standard to determine the efficiency of extraction and possible reaction of the model compound with the medium.

Molecular Weight Separation of Cell-Free Filtrate. A culture filtrate was passed through ultrafiltration membranes with molecular weight cutoffs of 10,000, 5000, 1000 , and 500 using an Amicon ultrafiltration apparatus.

Analytical Instrumentation. A HP 5880 gas chromatograph with a flame ionization detector was used to determine the amount of degradation for each model compound . The GC was equipped with a DB-5 30m column and appropriate flow rates were utilized as recommended by the manufacturer. The temperature program used was as follows: 50° C for 1 minute, 50-250° C at 8°/min, and 250° C for 10 minutes. The temperature of the injection port was 250° C and the temperature of the detector was 275° C. A HP 5985 GC/MS/DS was used in the electron impact mode at 70 eV to identify specific degradation products. The same type of column and temperature program were used. The temperature of the source was 200° C, the interface temperature was 275° C, and the electron multiplier voltage was 2500 eV.

RESULTS

Intact Organism. The gas chromatograms obtained from the degradation of benzylbenzoate and methoxybenzophenone by *C. versicolor* are illustrated in Figure 1. Figure 1a is a chromatogram of a 100 ppm standard of benzylbenzoate and 1b is a chromatogram after degradation by the intact organism. Figure 1c is a chromatogram of a 100 ppm standard of methoxybenzophenone and 1d is after degradation. These results clearly indicate that both compounds were degraded by the total organism. The data obtained from the degradation of model compounds by the intact organism is summarized in Table 1.

The extracts were analyzed by GC/MS. For benzylbenzoate, the primary degradation products included benzyl alcohol and benzoic acid.

Cell-Free Filtrate. The results for the degradation of benzylbenzoate, methoxybenzophenone, phenylbenzoate, and benzyl ether by the cell-free filtrate of *C. versicolor* are summarized in Table 1. Phenyl

benzoate was degraded the most; methoxybenzophenone was degraded only slightly. This may reflect the difference in reactivity of the two compounds was well as the selectivity of the enzymes.

Analysis of the degradation of anthracene by the cell-free filtrate indicated the presence of 9.10-anthracenedione (anthraquinone) as a degradation product in about 10% yield. Since anthracene can be oxidized to anthraquinone by a number of non- microbial mechanisms, it is important to note that the controls showed no indication of any quinones or any other degradation products.

Cell-Free Enzyme. For benzylbenzoate, the amount of degradation after one day was approximately 50% but methoxybenzophenone was degraded very little. It should be noted that 85-90% of the model compounds were recovered by extraction from the buffer solution, thereby eliminating reaction with the buffer as a possibility for model compound degradation. The results are summarized in Table 1 and are corrected for extraction efficiencies.

Molecular Weight Separation of Cell-Free Filtrate. The molecular weight separations indicated no reaction with the model compounds with the <10,000 MW fraction while the >10,000 MW fraction completely degraded the model compounds. It is interesting to note that the <10,000 MW fraction is primarily responsible for the biosolubilization of coal.

DISCUSSION

The results obtained from degradation studies of oxygen-containing compounds by *C. versicolor* and its extracts, shown in Table 1, indicate that the two compounds tested with the intact fungal organism were completely degraded. Complete degradation refers to no recovery of model compound. We can probably assume that the other two would also be totally degraded, since we have not yet found a simple compound that will survive long-term exposure to the intact fungus. The ease of degradation with the cell-free filtrate appears to be in the order: phenylbenzoate > benzylbenzoate > benzyl ether > methoxybenzophenone. Esters and ethers that are activated by aromatic rings appear to be susceptible to the fungal extract; however, aromatic ketones are not affected by the extract. From the limited results we have obtained from the isolated enzyme, it appears that the activity may parallel the cell-free filtrate. When

the cell-free extract was tested with the model compounds indole, dibenzothiophene, and bibenzyl, no degradation with the enzyme was noted; however, exposure of these compounds to the intact organism resulted in complete degradation.

Our results with anthracene indicate that its degradation by *C. versicolor* may occur through a radical cation mechanism similar to that proposed for trichlorophenol by *P. chrysosporium* (7). Related research has shown that there is a relationship between the ionization potential of the polycyclic aromatic hydrocarbon and the amount of degradation observed (8). Horseradish peroxidase has been shown to degrade polycyclic aromatic hydrocarbons to quinones, but only in the presence of hydrogen peroxide (9). It is of interest to note that our cell-free filtrate tests positive for peroxidase, although no peroxide is added to any of our biodegradation experiments.

The results of the molecular weight separations of the cell-free filtrate by ultrafiltration indicate the possibility of at least two mechanisms taking place during the biosolubilization process, one of which may be hydrolysis. The >10,000 MW fraction has been very reactive with the model compounds we have tried. The <10,000 MW fraction, where most of the biosolubilization occurs, has been found to be fairly unreactive toward most of the model compounds. Some preliminary results indicate the p-methoxyphenol may be a good substrate for this fraction.

Other preliminary results of further molecular weight separations indicate that the fraction of cell-free filtrate responsible for the majority of the biosolubilization activity is between 500 and 1000 MW. We are presently trying to characterize this fraction to determine the source of biosolubilizing activity.

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ACKNOWLEDGMENT

Table 1. Percent Model Compound Degradation by Coriolus Versicolor and its Cell-Free Isolates

Compound	Total Organism	Cell-Free Filtrate	Cell-Free Enzyme
benzylbenzoate	100 (a)(b)	56	55
methoxybenzophenone	100 (b)	1	2
dibenzothiophene	100 (c)		
phenylbenzoate		100	
indole	100 (c)		
bibenzyl	100 (c)		
benzyl ether		45	

(a) 100% = no model compound detected after incubation
(b) incubated one week with total organism
(c) incubated three weeks with total organism

Figure 1. Gas chromatograms of (a) benzylbenzoate, (b) after degradation, (c) methoxybenzophenone, and (d) after degradation by *C. versicolor*.
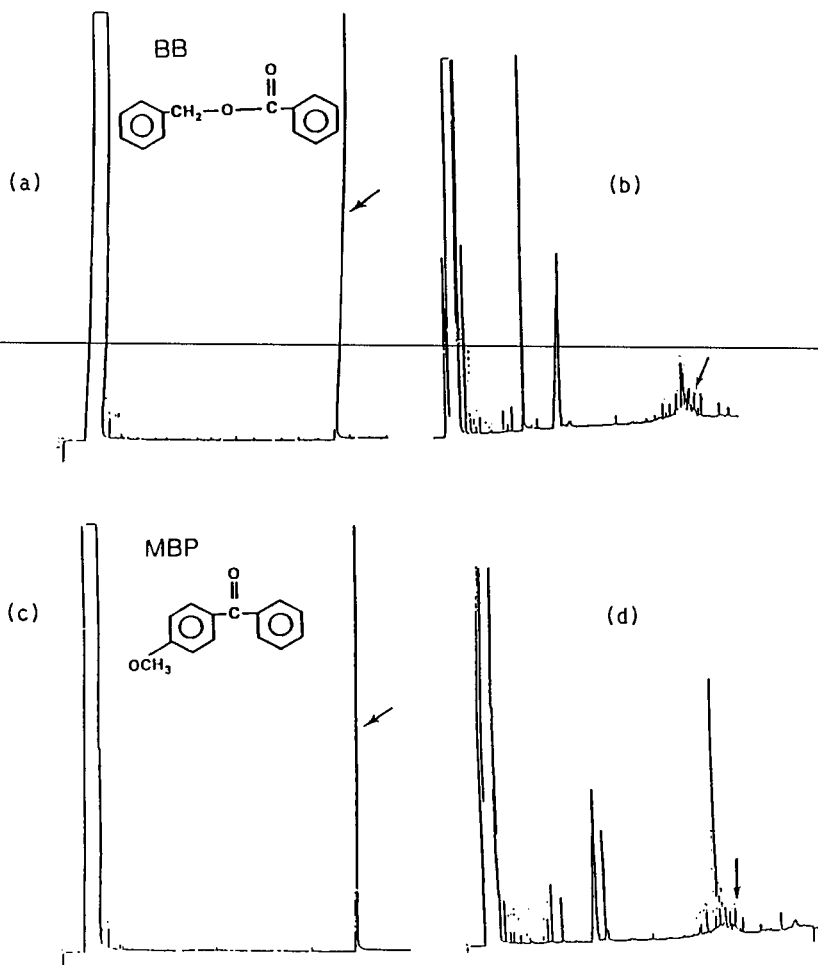
SERIAL BIOLOGICAL CONVERSION OF COAL INTO LIQUID FUELS

by

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ABSTRACT

Recently, several microorganisms have been shown to be capable of directly solubilizing low rank coals. This bioextract has a high molelcular weight and is water soluble, but is not useful as a liquid fuel. This paper presents the results of studies to biologically convert the solubilized coal into more useful compounds. Preliminary experiments have been conducted to isolate cultures for the serial biological conversion of coal into alcohols. Coal particles have been solubilized employing an isolate from the surface of Arkansas lignite. Natural inocula, such as sheep rumen and sewage sludge, are then employed in devloping cultures for converting the bioextract into alcohols. This paper presents preliminary results of experiments in coal solubilization and bioextract conversion.

INTRODUCTION

Microbial solubilization of coals and lignites is being developed as an alternative method of liquid fuels production. The biological approach under study offers the potential of significant cost-savings by converting solid coal to a liquid product, with minimal loss in total energy content, at near ambient conditions of temperature and pressure. The phenomenon of coal utilization and liquefaction by fungal and bacterial species was first reported in the early 1980's [1,2]. A number of strains of fungi and filamentous bacteria are now known to interact with low-rank coals, via extracellular processes, to yield a darkened medium when grown in submerged culture [3,4], or dark droplets of liquid on the coal's surface when grown on agar surface culture [1,3,5].

The coal liquid produced by bioliquefaction is a mixture of water soluble, polar organic compounds with relatively high molecular weights. Ultrafiltration and gel permeation chromatography have shown that the molecular weight is in the range of 30,000 to 300,000 [3]. The chemical

structure is extremely aromatic, with a large number of hydroxyl groups. Because of the low relative volatility, analysis to quantify the product by mass spectrophometry or gas chromatography has been difficult.

While this technology has great potential, there are a number of serious problems to be solved. In the absence of water, or suitable solvent, the product is a solid. Although the solubilized product has a reasonably high energy content and may be useful as a combustion fuel, it is not suitable as a transportation fuel. Also, most of the organisms require expensive sugars and media for growth over a period of about two weeks. Cheap media and fast growing organisms will be required for commercial application. Another serious economic problem relates to the requirement for pretreatment to achieve high yields. Highly oxidized lignites, such as leonardite, can be converted almost entirely. Higher rank coals must be chemically oxidized before significant liquefaction occurs in a reasonable time (6,7). Chemical treatment is likely to be cost prohibitive.

Research at the University of Arkansas has led to the isolation of a bacterium, as yet unidentified, that is fast growing in cheap mineral salts media and that converts coal into liquid and floculate in a few hours in submerged culture. High rank coals are converted without pretreatment. This rod-shaped bacterium is not a _Streptomyces_ and is likely a previously unknown strain with coal or lignin activity. Further study is required to develop and quantify coal conversion with this organism.

It is highly unlikely that any single organism will be able to completely liquefy coal to low molecular weight fuels. However, it is likely that organisms can be utilized to upgrade the initial microbial products to useful fuels. Such a second-stage conversion should probably be anaerobic to avoid further oxidation of the product. The evaluation of serial biological conversion of coal to liquid fuels is currently underway. This paper presents a brief summary of progress to biologically produce low molecular weight liquid fuels from coal.

MATERIALS AND METHODS

Substrate

The solubilized lignite used as the substrate for the anaerobic bacteria was obtained from submerged culture experiments. An organism isolated from an Arkansas lignite sample was used to solubilize the coal.

The solubilization was carried out in a 1.5 liter Biostat M stirred-tank fermentation system from B Braun Instruments. The fermenter was equipped with pH and temperature control as well as a dissolved oxygen probe. The temperature was maintained at 28°C and the agitation rate was 150 rpm.

The lignite surface culture was allowed to grow for 3 days on a media consisting of 0.5 percent glucose and 0.5 percent peptone. After 3 days, 9g of 8-20 mesh Arkansas lignite were added. Within 24 hours, 35 percent of the lignite was solubilized. The solubilized lignite was collected by filtration and precipitated by adjusting the pH of the solution to 1.0 with acid. The

precipitate was washed and dried and then redissolved in distilled water by adjusting the pH to approximately 6.5. Portions of this solution were then used as substrate in the attempts to produce alcohol fuels.

Media

The original culture media contained yeast extract (Difco), 0.1g; B-vitamins (Wolfe's), 1 ml; KH_2PO_4, 0.6 mg; $(NH_4)_2SO_4$, 0.6 mg; NaCl, 1.2 mg; $MgCl_2 \cdot 6H_2O$, 0.4 mg; $MgSO_4 \cdot 7H_2O$, 0.3 mg; lignite, 0.03 g, added to 100 ml of deionized water, and adjusted to pH 7 with NaOH.

Media for transfers from the original cultures contained yeast extract (Difco), 2.0 g; α-D glucose (Aldrich), 5.0 g; B-vitamins (Wolfe's), 10.0 ml; $ZnSO_4 \cdot 7H_2O$, 0.5 mg; $MnCl_2 \cdot 4H_2O$, 0.15 mg; H_3PO_3, 1.5 mg; $CoCl_2 \cdot 6H_2O$, 1.0 mg; $NiCl_2 \cdot 6H_2O$, 0.1 mg; Na_2MoO_4, 0.5 g; $FeCl_2 \cdot 4H_2O$, 7.5 mg; Na_2SeO_3, 0.05 mg; KH_2PO_4, 0.5 g; $MgCl_2 \cdot 6H_2O$, 0.33 g; NaCl, 0.4 g; NH_4Cl, 0.4 g; $CaCl_2 \cdot 2H_2O$, 50 mg; rumen fluid (filtered and sterilized in autoclave), 100 ml; lignite, 0.3 g, per liter of water, and adjusted to pH 7 with NaOH. The control medium was of the same composition without lignite.

Media solutions were made anaerobic by briefly boiling, then cooling under 80% N_2/20% CO_2; the gases passed over heated copper to remove any oxygen. Anaerobic media was transferred to 100 ml stoppered serum bottles and autoclaved at 15 psig for 20 minutes. Just prior to inoculation of media, $Na_2S \cdot 9H_2O$, 0.05%, was added to each media bottle to lower the oxidation-reduction potential. Resazurin was not added as an indicator of anaerobiosis as it would interfere with spectrophotometric readings; however, experience with the anaerobic techniques used here have shown them to be reliable.

Cultures

Rumen has been used a number of times as source of bacteria capable of breaking down complex structures (8,9,10). Cultures were started with fresh rumen contents from cow and sheep. Only sheep rumen cultures showed lignite-degrading potential and were studied further. Following growth of serial dilutions of a sheep rumen culture, the dilution tube showing the greatest color change from baseline as measured on the spectrophotometer by its absorbance at 580 nm was transferred to fresh media; subsequent sheep rumen cultures were all derived from this dilution tube. Cultures were started generally with a 10% inoculum to fresh media. Incubation was at 37°C, shaking at 100 rpm.

A mixed culture derived from sheep rumen which appeared to degrade lignite at 0.03 g% was streaked onto plates; solid media had the same composition as the liquid media with glucose, with the addition of 2.0 % agar (Difco). Plates were poured, and all inoculating was done inside an anaerobic chamber. Inoculated plates were incubated at 37°C in an anaerobic jar (Oxoid). Three different colonies were transferred to solid media in slant tubes and later to liquid anaerobic media in flasks.

Microscopically, the isolated cultures did not look pure. One showed growth of large rods with endospores, but it appeared there were also smaller

rods present. The other two cultures showed growth of at least two types of rods - a long, slender rod, and a shorter, more refractile one.

Liquid samples were also analyzed on a spectrophotometer. A standard plot of optical density or absorbance at 580 nm versus lignite concentration using dilutions of lignite in water was made and a linear plot was obtained (Figure 1); the darker the solution, the higher the liqnite concentration. Thus, it was concluded that spectrophotometry could be used to follow the lignite utilization of the culture. Liquid samples were centrifuged at 10,000 x g for two minutes. One ml of supernatant was diluted with 1 ml of distilled water. Readings of absorbance at 580 nm were taken on a Bausch and Lomb Spectronic 21. Control media served as reference for corresponding lignite media samples.

Results

Of the initial mixed cultures of sheep rumen and cow rumen, the sheep rumen culture was the only one to show a substantial visual decrease in color during fermentation with a decrease in the absorbance at 580 nm from 0.305 to 0.047 as seen in Tables 1 and 2, indicating a decrease in the lignite concentration of approximately 90 percent.

The three cultures derived from the original sheep rumen culture were also tested for their ability to degrade solubilized lignite based on the decrease in absorbance at a wavelength of 580 nm of a culture containing 0.03 percent solublized lignite. The results of this test are presented in Table 3. As seen in Table 3, culture 2 appears able to degrade solubilized lignite better than cultures 1 and 3.

Due to the apparent ability of culture 2 to degrade solubilized lignite, it was chosen for further work. This culture was inoculated into five culture tubes with the media for transfers described above except the solubilized lignite concentration varied from 0.03 percent to 0.25 percent. The absorbance at 580 nm was monitored on each tube with time to determine the affect of solubilized lignite concentration on the degrading ability of the culture. The results of these experiments are shown in Table 4. Based on these results it appears that solubilized lignite concentrations of 0.1 percent or higher inhibit the cultures ability to degrade the lignite.

Further work is being done with these cultures to determine the ability to produce lower alcohols and organic acids. At this time, none of the cultures have shown the ability to produce substantially higher quantities of ethanol, propanol, butanol, acetic, propionic or butyric acids from solubilized lignite when compared to a control culture without solubilized lignite. Work is continuing, however, on the analysis of these experiments to better determine what compounds are being produced.

CONCLUSIONS

Preliminary results have been obtained in the second step of the serial biological conversion of coal to liquid fuels. Solubilized lignite, derived from the action of a lignite surface culture on Arkansas lignite, was used as the substrate in anaerobic culture with organisms derived from sheep rumen.

In mixed culture, the potential for degrading lignite was shown by the decrease in color and absorbance at 580 nm during fermentation. Analysis of the fermentation products of these cultures, however, has not shown substantial production of lower alcohols. The search for an anaerobic organism capable of producing higher quantities of alcohols is continuing. Work is also being conducted in an attempt to find aerobic organisms capable of degrading solubilized lignite to compounds which can then be converted to alcohols by anaerobic organisms.

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Table 1

Optical Density or Absorbance at 580 nm of
Original Cow Rumen Culture

days	A_{580} (Sample)-(Control)	Control
0	0.202	0.360
2	0.310	0.198
4	0.293	0.205
6	0.280	0.280
10	0.285	0.208

Table 2

Optical Density or Absorbance at 580 nm of
Original Sheep Rumen Culture

days	A_{580} (Sample)-(Control)	Control
0	0.305	0.213
1	0.278	0.210
2	0.235	0.238
3	0.158	0.263
6	0.047	0.335

Table 3

Absorbance at 580 nm of Cultures Derived from Sheep Rumen

Culture	day	A_{580}
1	0	0.328
	10	0.240
2	0	0.328
	10	0.170
3	0	0.328
	17	0.225

Table 4

Optical Density or Absorbance at 580 nm of Culture With Varying Lignite Concentrations

Lignite Concentration	days	A_{580} (Sample)-(Control)*	Control**
0.03%	0	0.263	0.310
	3	0.085	0.277
	7	0.057	0.267
0.05%	0	0.344	0.310
	3	0.203	0.277
	7	0.033	0.267
0.10%	0	0.740	0.310
	3	0.788	0.277
	7	0.768	0.267
0.15%	0	1.050	0.310
	3	1.013	0.277
	7	1.033	0.267
0.25%	0	--***	0.310
	3	--	0.277
	7	--	0.267

  *   average of two samples
 **   average of three samples
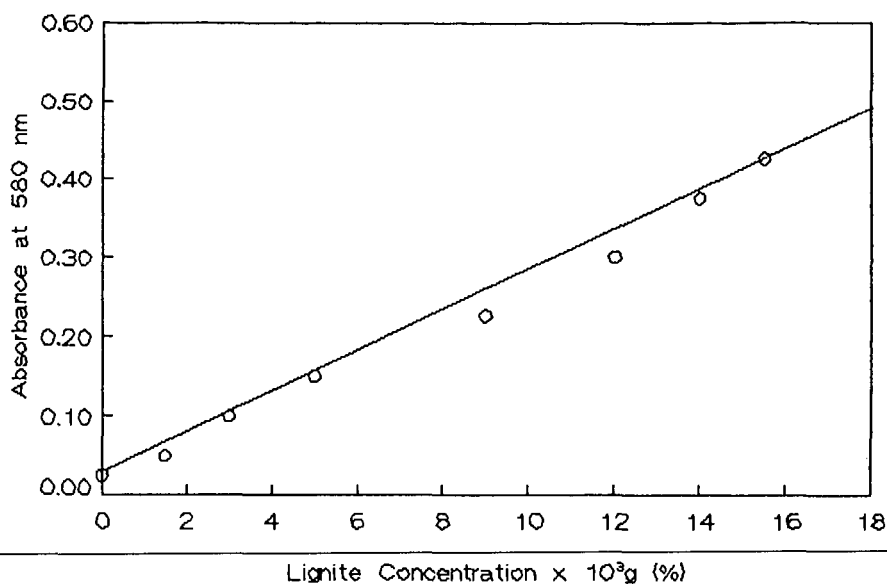***   sample reading was off instrument's scale

Figure 1. Calibration curve for solubilized lignite concentration using absorbance.

RECENT PROGRESS IN CELL-FREE SOLUBILIZATION OF COAL

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ABSTRACT

Low rank coal has been solubilized using cell-free filtrates separated from cultures of Polyporus versicolor. Solubilization has been obtained with neat filtrates and with fractions collected from the neat filtrates after gel permeation chromatography. The coal solubilizing enzymes have been collected in enriched fractions with gpc. This increased relative purity has allowed the determination of the average molecular weight of this enzyme by gel permeation chromatography and by polyacrylamide gel electrophoresis. Rates of coal solubilization are dependent on the size of coal particles, mass of coal, temperature, pH, concentration of the cell-free filtrate, and the concentration of several inorganic ions.

INTRODUCTION

The focus of experimentation at the beginning of this project was to produce a solubilized product from coal which had been exposed to the fungus Polyporus versicolor. Initially, Polyporus was routinely grown in dishes containing Sabouraud maltose agar and incubated at 30°C. Leonardite was routinely solubilized by adding small pieces directly to the surface of the growing mycelium. In a second stage, Polyporus was grown in a liquid medium consisting of Sabouraud broth. After a suitable period of fungal growth, the hyphae were separated from the culture medium by filtration and aliquots of the cell-free broth were added to samples of leonardite. The general characteristics of the observed (cell-free) solubilization were reported in the open literature (1). The research presented here has the ultimate aim of isolation and purification of the active agent(s) in the solubilization process, which are present in the neat cell-free broth. Ultimate success does, of course, require a knowledge of the presence of those agents and their relative concentrations. Thus, this paper reports the optimization of the characteristics of the solubilization process, and a standard set of conditions for the testing of the solubilization phenomena. From this base, a discussion of the progress towards isolation and purification of the species involved in the solubilization process is presented.

OPTIMIZATION AND STANDARDIZATION OF THE SOLUBILIZATION PROCESS.

Having identified the scope of the solubilization process, it became necessary to determine an optimal set of conditions for the study of the process itself. A standard set of conditions is necessary in order to develop an assay for the solubilization ability of the broth, with some form of assay being required in order to compare the activity of various samples of broth with each other. This set of conditions is also necessary to be able to systematically avoid any inhibitors of the process.

An initial series of tests showed that some ionic strength in the solubilization medium was helpful in improving the solubilization action of the Polyporus broth. While nitrate, perchlorate, and acetate showed little effect at pH = 5.5 (even

though acetate has buffering capacity at this pH), the presence of phosphate
(Figure 1) and, to some degree, chloride, did influence the solubilization of
coal. The explanation at the time centered around the ability of these latter two
bases to coordinate ferric ion, especially phosphate. From this work, a
standardized buffering system of 0.050$\underline{M}$ phosphate and 0.050$\underline{M}$ acetate (all sodium
salts) was established.

Using this medium, it was determined that the broth did not simply act as a
catalyst for coal dissolution, but that the broth had a capacity, and that this
capacity could be measured (Figure 2). This was the first standardization
procedure for the solubilization ability of the broth. Knowing that the action of
the broth was not being limited by its inherent capacity, and the fact that the
constant medium being used and the constancy of the procedures was dramatically
reducing the scatter in the data which was being obtained, it was possible to
obtain a solubilization yield of the broth for the first time. This was
accomplished by comparing the lyophilized bioextract from the agar plates against
the liquid culture solubilizations. A 70% yield of coal solubilization was
obtained, and that particular batch of broth had a capacity of 0.32mg/mL at
pH = 5.5. At pH = 5.25, the yield was only 32% but the capacity was above
1.5mg/mL.

In a parallel investigation, an increasing amount of the broth was added to a
fixed amount of coal. Figure 3 shows that the absorbance of solubilized coal is a
function of the concentration of broth at low broth concentrations, and becomes
independent of the amount of broth at higher concentrations. It should be noted
that undissolved particles were present in all the test mixtures. This supports
the concept of enzyme capacity discussed above.

The pH effect mentioned above prompted the generation of a pH profile (Figure 4).
The maximum effect appears between pH = 5.3 and pH = 5.5. It is interesting to
note that the activity does not rapidly fall to zero as pH decreases, but appears
to fall to zero as pH increases by one unit. This rapid drop at higher pH is at
least partially due to the much higher background of solubilization by the buffer
at higher pH values.

In an attempt to understand the nature of the active agent in the broth, and,
thus, to better be able to handle the system, ascorbic acid (a reducing agent) was
added to inactivate any oxidative enzymes which might be present in the broth.
The addition of ascorbic acid decreased the effectiveness of the broth by 90%,
while the addition of oxygen after the ascorbic acid (to oxidize the ascorbic acid
and show that the oxidized ascorbate was not responsible for the inactivity of the
broth) restored the activity. The addition of oxygen to the broth alone showed a
slight increase in the activity of the broth.

A temperature profile of the rate of solubilization activity was developed.
Figure 5 shows a steady increase of the rate of cell-free solubilization with
increasing temperature until 60°C after which the rate of solubilization turns up
very quickly. The active agent for solubilization must be very rugged, and any 3-
dimensional property of the agent must not be important in solubilization.
Another critical point in the analysis is the presence of cations which are poor
Lewis acids (i.e. cations which tend to be "soft" and basic in solution). It was
noticed early in 1987 that virtually all the iron which the coal contained was
brought into the solution phase during the solubilization process. When various
eluents were being used in the HPLC separation of the broth, it was noticed that
the presence of added Fe(III) as well as cations such as ammonium ion and

tetraethylammonium ion inhibited the solubilization process. This initial
nuisance has become a potential analytical probe for the activity of the broth.
In Figure 6, the intersection of the negative slope and the horizontal baseline
is a direct function of the concentration of active agent, since the iron content
eventually stops the solubilization process. Thus, for the same coal sample and
time of contact, the slope of the absorbance vs. Fe(III) line should remain
constant as the x-axis intercept changes to reflect the activity of the particular
enzyme sample.

Furthermore, the active agent has a very strong binding constant toward iron,
which presents itself as an irreversible process in the simple sense. This is
seen when EDTA is added to a sample of coal, broth, and excess iron. If the iron
is added to the broth before the EDTA, the broth is inhibited under conditions
where the EDTA is able to coordinate all the iron. If the EDTA is added before
the broth, it coordinates to the iron and the solubilization continues normally.
Not all cations act in the same manner as Fe(III). Figure 7 shows the effect of
the addition of ammonium ions on the solubilization process. As seen in the
Figure, the concentration of NH_4^+ needed for a loss of enzyme activity equivalent
to that seen in the Fe(III) system is much greater. The far greater effectiveness
of Fe(III) in inhibiting the solubilization process implies that the binding
constant of ammonium ion with the active enzyme is clearly much lower than that
with Fe(III). Even at 0.5$\underline{M}$, the ammonium ion presence has not totally inhibited
the solubilization process, although it is significantly decreased.

ISOLATION OF THE ACTIVE AGENT(S) IN THE SOLUBILIZATION PROCESS.

With a set of standard analytical procedures in place, the process of separating
the active agent(s) from the broth was begun.

Using a C_{18} column with the standard phosphate/acetate buffer as eluent, two of
the nine discernable peaks in the chromatogram showed activity toward coal (Figure
8). In order to further resolve these peaks, the eluent polarity was varied from
that of methanol to 5% (w/w) NaCl. Resolution increased with polarity, but the
peaks of interest always partially overlapped the void volume. Thus, another
separation technique was necessary. Even so, gel electrophoresis separations on
the broth produced patterns of protein which mirrored those of the C_{18}
chromatography, indicating that the number of large molecules from which a
separation had to be effected was not enormous.

Anion exchange initially showed some potential. The compound(s) of interest
apparently have weak bases which have pK_a's in the pH = 5.5 region, since the
placement of peaks coming off the anion column was quite sensitive to pH and [Cl⁻]
in this pH region. The work carefully led to ever further refinements of
procedure from step gradients to gradient elution through various concentrations
of Cl⁻ and H⁺.

The difficulty with all these methods is the high [Cl⁻] which was part of the
anion-exchange process. Since coordinating agents which might ordinarily be of
use in removing the Cl⁻ are not available to the work because of the inhibition of
the system, the method of separation turned to exclusion chromatography. With an
analytical gel permeation column (Waters Protein-Pak 125), the active solubilizing
agent can be isolated to a single fraction of the chromatogram (Figure 9). A
similar pattern of separation can be accomplished on a preparative gpc column
(Sephadex, G-25-150) as shown in Figure 10.

In order to determine the molecular weight of the active fraction, the analyticalgpc column was used with six compounds of known molecular weight. The active fraction eluted from the column in an elution volume of 11.9 mL (Figure 11). This corresponds to an average molecular weight of 25,000 Daltons.

FUTURE EXPERIMENTATION

Experiments aimed at purification of the proteins responsible for solubilization of coal will be continued, with the goal of the formulation of a large-scale process of enzyme separation.

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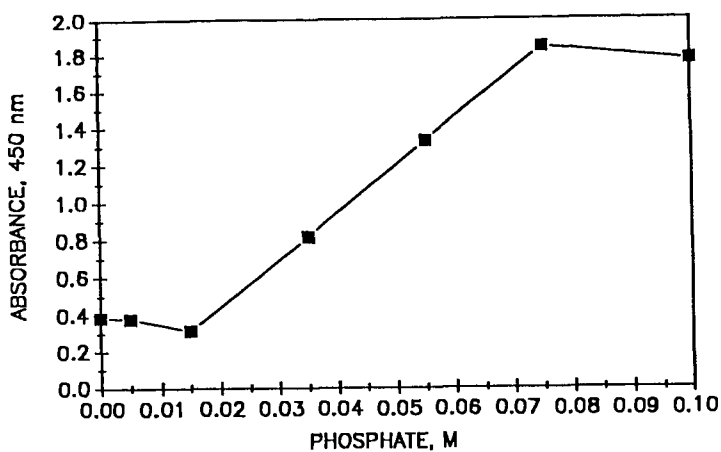
ACKNOWLEDGEMENT

Figure 1. Effect of phosphate concentration on cell-free solubilization of leonardite coal.
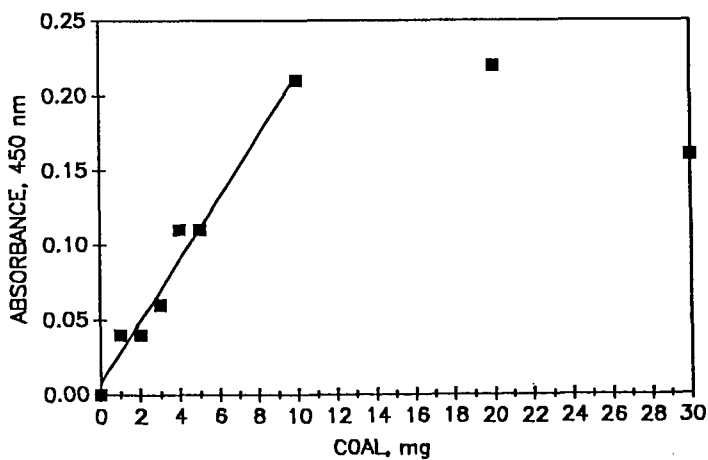

Figure 2. Effect of mass of coal added to a constant amount of cell-free filtrate on extent of solubilization of leonardite.
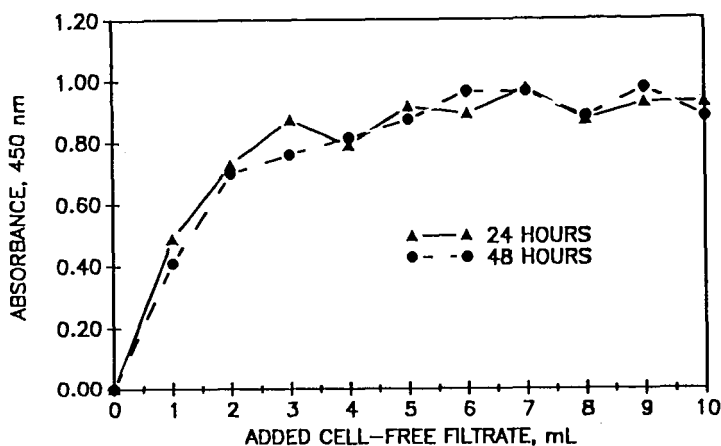
Figure 3. Effect of various amounts of cell-free
filtrate added to a constant amount of leonardite on
extent of solubilization of leonardite.
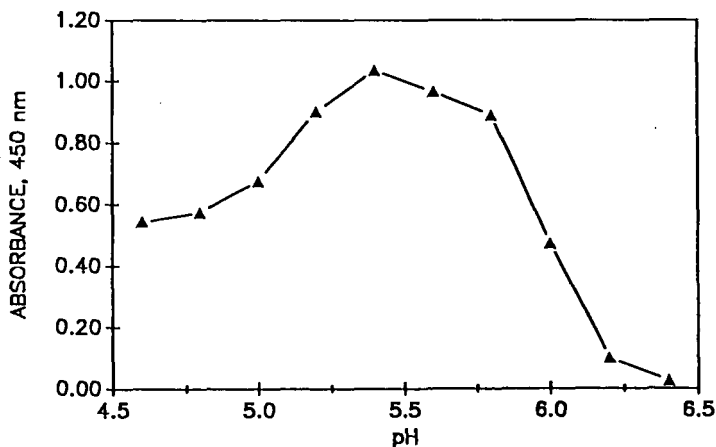
Figure 4. Effect of pH on activity of the cell-free
filtrate separated from the SMB in which _Polyporus_ had
grown.
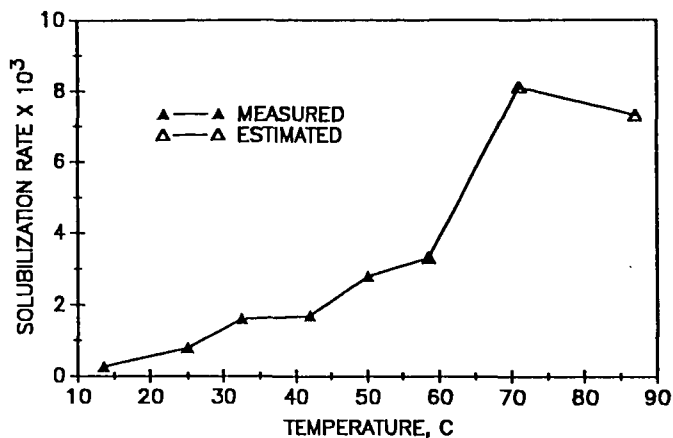
Figure 5. Effect of temperature on the rate of cell-free solubilization of leonardite coal.
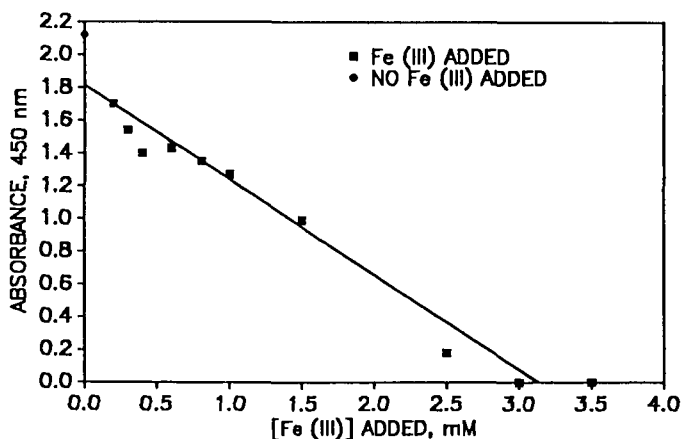
Figure 6. Inhibition of cell-free solubilization of leonardite coal by addition of Fe(III) to the filtrates. The inhibition can be reversed by mixing EDTA with the Fe(III) before addition of the iron solution to the filtrates.
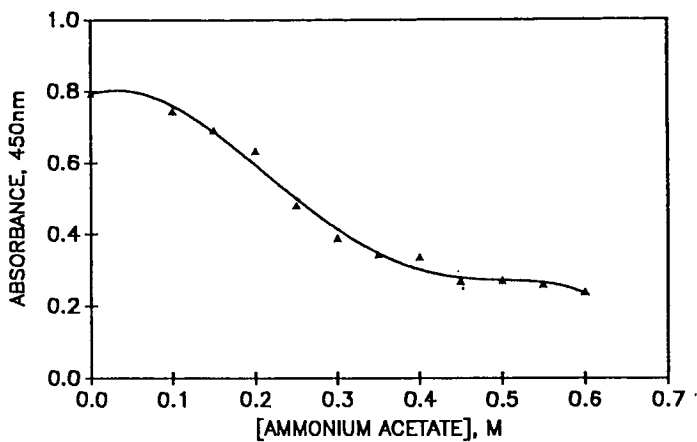
Figure 7. Inhibition of cell-free solubilization of leonardite coal by addition of ammonium ion to the filtrates.
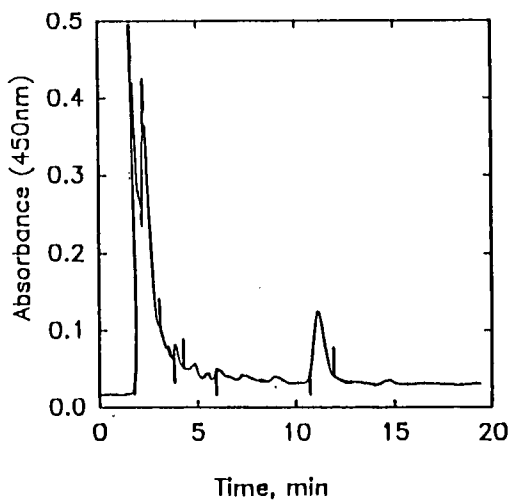
Figure 8. Separation of cell-free filtrate from Polyporus on a C₁₈ column.
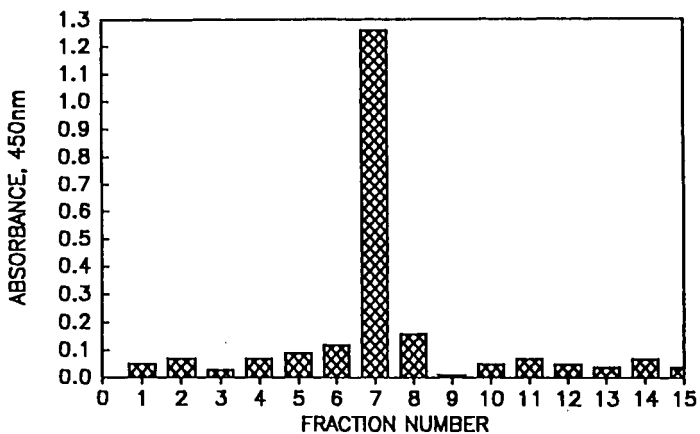
Figure 9. Fractionation of the neat cell-free filtrate on a gel permeation column. The leonardite-degrading activity has been separated into a single fraction.
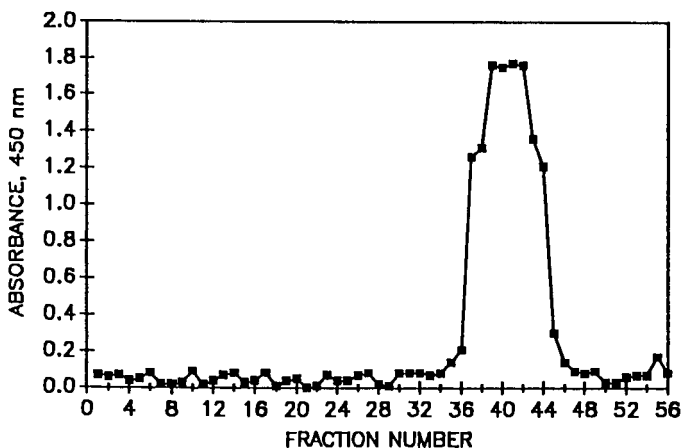
Figure 10. Separation of the coal solubilizing enzyme on a preparative column (1.5 cm X 25 cm) packed with Sephadex G-25-150 resin.

Figure 11. Molecular weight determination of the coal-solubilizing enzyme.

BIOSOLUBILIZATION OF COAL IN AQUEOUS AND NON-AQUEOUS MEDIA

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ABSTRACT

Biocatalytic solubilization of coal can be achieved by microorganisms in aqueous
medium or by enzymes in aqueous or organic media. In these systems, coal is
converted to a liquid product through a variety of oxidative and/or hydrogena-
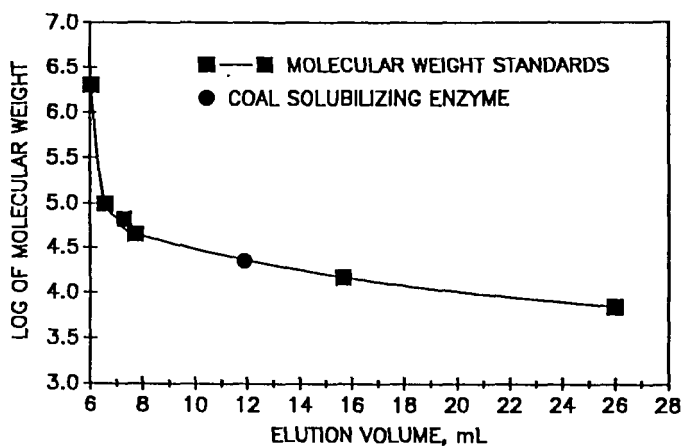tive reactions.

The mechanism and control of coal solubilization activity have been studied in
organisms associated with coal in nature. The solubilization apparently
involves catalysis by alkaline cell metabolites. The aqueous product of this
microbial action may be a suitable substrate for other biological interactions
such as biogas production by methanogenic organisms.

Enzymatic coal solubilization has been demonstrated in both shake flasks and
fluidized-bed reactors. The process has been carried out under both anaerobic
and aerobic conditions, with aqueous and hydrous organic solvents. The solubi-
lization product of the anaerobic process is much less polar than that from
microbial solubilization.

INTRODUCTION

The application of biotechnology to the utilization of low-ranked coals has
become the focus of intense research activity in recent years. Biological
systems -- whole organisms or fractions thereof -- have been shown to catalyze a
variety of industrially significant reactions. These systems have potential
utility in the upgrading and/or transformation of coal.

Biocatalysis generally occurs under mild, "physiological" conditions, e.g. at
relatively low temperatures and pressures, and at pH near neutrality.
Biological treatments of coal could therefore be conducted under mild reaction
conditions relative to those under which conventional thermo/chemical processes
are carried out. Biocatalysts characteristically demonstrate high specificity
with respect to the products generated. Biological treatment of coal would thus
promote the formation of specific products, including perhaps products with
higher fuel value than that of the original substrate.

Biotechnology can be applied to various aspects of coal processing. The appli-
cation which has received the greatest attention is the removal of contaminating
heteroatoms, such as sulfur, from low-ranked coals by microorganisms. The use
of biocatalysts to modify coal's carbon skeleton is, however, a rapidly deve-
loping area of great potential importance. Research at Oak Ridge National
Laboratory has focused on the biotransformation of lignite and subbituminous
coals by microorganisms or by isolated biochemical catalysts (enzymes). The

synthesis of liquid products from coal by biological systems, i.e. coal biosolu-
bilization, is the subject of this exploratory research. This work will form a
basis for the development of technologies for the production of clean-burning
liquid or gaseous fuel products from coal substrates. The results of current
initiatives in this area are summarized below.

Biosolubilization of coal in aqueous media

The solubilization of coal by microorganisms was first reported in 1982 by Cohen
and Gabrielle (1). These and other workers noted the production of liquid
droplets from coal, associated with the growth of mycellial organisms on the coal
surface. Organisms competent to solubilize coal were isolated from coal in the
environment (2) or in the laboratory (3). It was subsequently shown that these
organisms solubilized coal when cultured on the surface of common, organic
microbiological media. Suitable coal substrates for this activity were leonar-
dite, lignites, and subbituminous coals. In the latter cases, the coals
required oxidative pretreatment either through natural weathering or by chemical
agents [hydrogen peroxide, ozone, nitric acid, etc.; (4)]. The product of this
microbial activity was a water-soluble mixture of oxidized compounds of moderate
molecular weight [30,000-300,000 daltons; (3)]. The material was enriched in
carbonyl and hydroxyl functions relative to the coal substrate, and was precipi-
table at pH 1. Its characteristics resembled those of humic acids, except for
its water-solubility.

Recent work (5,6) in this laboratory has sought to determine the mechanism by
which microorganisms solubilize coal in vivo. Superior isolates (fungi) have
been cultivated in a defined growth medium in both surface culture on agar and
submerged culture in liquid medium. These culture methods simulate fixed-bed
and fluidized-bed bioreactor configurations respectively, which have been pro-
posed for use with this technology (Figure 1). The defined media developed for
use in this work consisted of inorganic salts, supplying the organism's mineral
requirements, plus a sole carbohydrate carbon source (5). These media support
coal solubilization in vivo (Table 1). The use of defined media has minimized
contamination of the liquid coal product with organic medium components, and
will contribute to further product analysis. product recovery has been expe-
dited by growth and solubilization under submerged culture conditions, i.e. in
shake flasks. use of this system has also permitted the development of a
spectrophotometric assay for coal solubilization, based on the appearance of
chromophoric material (absorbing in the 420-450 nm spectral range) in cultures
incubated with coal. Its spectral characteristics were identical to those of
the material formed by the nonbiological action of alkali on coal.

The solubilization of coal by dilute alkali had been demonstrated previously,
and had been implicated in the activity in vivo (6). Specifically, it was
thought that microbial coal solubilization occured as a fortuitous consequence
of pH increases associated with growth. Evidence in support of this conclusion
had been obtained in alkaligenic systems substantially contaminated with protein
and other basic components. In the present work, activity was detected in an
acidogenic system of known biochemical composition (Figure 2). These data sup-
port an involvement of alkaline catalysis in the microbial activity. The data
suggest further that the proposed alkaline catalyst is produced by cultures in
specific response to the presence of coal.

The liquid product generated by fungal action on coal may have some utility as

a feed stream for the biological production of other combustible fuels. Possible products are biogas (methane) and fuel alcohols.

Methane production from partially-oxidized compounds analogous to coal substructures has been demonstrated elsewhere. Based on this knowledge, a two-step process for conversion of coal to methane has been proposed. Coal solubilization would be carried out by aerobic microorganisms as described above. Subsequent biogas production would be accomplished through the action of anaerobic organisms. Preliminary tests have been conducted in order to determine the feasibility of the proposed process.

Microbial consortia isolated from a municipal sewage treatment plant or from a coal fly ash settling pond were incubated with biosolubilized coal under anaerobic conditions. The liquid coal product was nontoxic at low concentration toward microbial growth or metabolism. After acclimation, methanogensis from methanol occurred with the same yield with or without the coal liquids present. Model compound studies were carried out with vanillin, which was shown to disappear from metabolically active cultures. A defined growth medium was designed for use with these methanogenic consortia. In that system, either vanillin or the coal liquid could serve as sole carbon source for growth and metabolism. Preliminary data from total organic carbon analysis with the coal liquid as the sole carbon source indicated at least partial degradation. These findings suggest that biosolubilized coal can be utilized by methanogenic consortia for biogas production, accomplishing a two-step conversion of coal to clean-burning gaseous product. These data also support the feasibility of using anaerobic microorganisms for other treatments of liquid coal product, possibly including the conversion of coal to fuel alcohol.

The feasibility of treating coal with enzymes in aqueous buffer has been explored. Preliminary trials were conducted with horseradish peroxidase and laccase from Pyricularia oryzae. These enzymes are a nonspecific oxidoreductase, transferring electrons from H_2O_2, and an oxygenase respectively. The reactions were carried out in shake flasks under aerobic conditions. A measurable increase in coal solubilization occurred when laccase was used, although the extent of solubilization was somewhat low (Table 2). Horseradish peroxidase also increased solubilization, but to a smaller extent. The material yielded by enzymatic solubilization was water-soluble, and of a polarity similar to that of the product of microbial action.

Biosolubilization of coal in non-aqueous media

Bioprocesses involving intact microorganisms generally are carried out in aqueous environments, i.e. growth media or buffers. Considerable excitement has been generated by the finding that isolated enzymes can be used in organic solvents to catalyze certain reactions (8). The bioprocessing of coal in nonaqueous media would favor the formation of nonpolar products, possibly including reduced compounds with high fuel value.

Oxidizing enzymes would be required to catalyze the various reactions involved in coal depolymerization. Additional treatment with reducing enzymes may result in the production of low-molecular-weight nonpolar compounds similar to those generated by nonbiological coal liquefaction. These enzymes would cause a net increase in hydrogen content and a reduction in oxygen content. Recovery of

these products would be facilitated if these reactions were carried out in a non-polar organic solvent. The use of enzymes in organic solvents is complicated by the need to maintain the enzyme within an aqueous microenvironment in order to support activity (8). Hydrous organic media are therefore used in these systems.

The potential for enzymatic coal solubilization in nonaqueous media was evaluated via tests with horseradish peroxidase, alcohol dehydrogenase from yeast, and/or bacterial hydrogenase. Preliminary experiments were performed under aerobic conditions (air) using peroxidase dissolved in hydrous dioxane. The extent of coal solubilization was somewhat increased by this treatment, and was much greater than that previously measured in buffer alone (data not shown). Subsequent tests were carried out with enzyme mixtures in dioxane or pyridine, under a reducing atmosphere of 100% hydrogen. Solubilization of a leonardite was substantially enhanced by enzyme treatment (Table 3). A much smaller positive effect was seen in tests with bituminous coal.

The liquid product from anaerobic coal solubilization in organic solvent was found to be soluble in benzene (data not shown). This product thus differed from that obtained by aerobic treatment with enzymes or intact microorganisms in aqueous systems with respect to polarity and solubility behavior.

Additional studies were carried out in a fluidized-bed bioreactor under aerobic conditions, in which coal was treated with peroxidase in hydrous dioxane. Solubilization was very rapid and was enzyme-dependent (Figure 3).

SUMMARY

Biological treatments with whole cells or isolated enzymes has the potential to yield useful products from low-ranked coals. These products may include clean-burning gaseous or liquid fuels. Coal solubilization is a common element in these conversions. Exploratory research indicates that the characteristics of the product(s) obtained are dependent on the choice of operating conditions, i.e. whether in aqueous or organic solvent, and in the presence or absence of air. The development of future bioprocesses for coal utilization must build upon these findings, to achieve the directed synthesis of desired products.

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Table 1. Coal solubilization by Paecilomyces TL1 (3; surface cultures)[a]

Medium[b]	Coal solubilization[c] (% weight loss)
Complex:	
Sabouraud maltose	12.4
Defined:	
Czapek's	25.9
Minimal I	[5.3]

[a]Cultures were grown at 30°C, 80% RH for 7 d. Coal was added (0.5 g) and cultures reincubated for 7 d under the same conditions.
[b]Media contained 0.1% malose.
[c]Controls (uninoculated medium) exhibit $\leq$5% weight loss. Weight loss measurements incorporate $\leq$20% error.

Table 2. Enzymatic solubilization of acid-treated subbituminous coal in the presence of 0.05 M phosphate aqueous buffer at pH 6.0

Aqueous constituents	Average weight loss (%)
Buffer only	2.5
Buffer + 1625 units of peroxidase/mL	4.4
Buffer + 700 units of laccase/mL	6.8

Table 3. Anaerobic solubilization of coal in a hydrogen environment at ambient temperature and pressure by enzymes in organic solvents[a]

Liquid phase	Type of coal	Enzymes	Weight loss of coal (%)
Dioxane + buffer	Lenordite	None	19.6
Dioxane + buffer	Lenordite	Mixed[b]	86.4
Dioxane + buffer	Bituminous	None	1.2
Dioxane + buffer	Bituminous	Mixed[b]	3.7
Pyridine + buffer	Bituminous	None	3.0
Pyridine + buffer	Bituminous	Mixed[b]	6.7
Pyridine + tetralin + buffer	Bituminous	None	4.6
Pyridine + tetralin + buffer	Bituminous	Dehydrogenase	6.0

[a]The tests were carried out for 48 h with 20 mL of liquid and 0.3 g of coal size-reduced to a range of -10 to +30 mesh in 50-mL shake flasks with a controlled gas environment in the flask headspace. When enzymes were used, they were introduced with an activity of 400 units/mL for peroxidase, 0.325 units/mL for hydrogenase, and 3640 units/mL for dehydrogenase. A 0.1 $\underline{M}$ aqueous phosphate buffer, pH 5.6, that constituted 5% (v/v) of the liquid solution was used.

[b]The enzyme mixture included peroxidase, hydrogenase, and dehydrogenase in equal weight proportions.
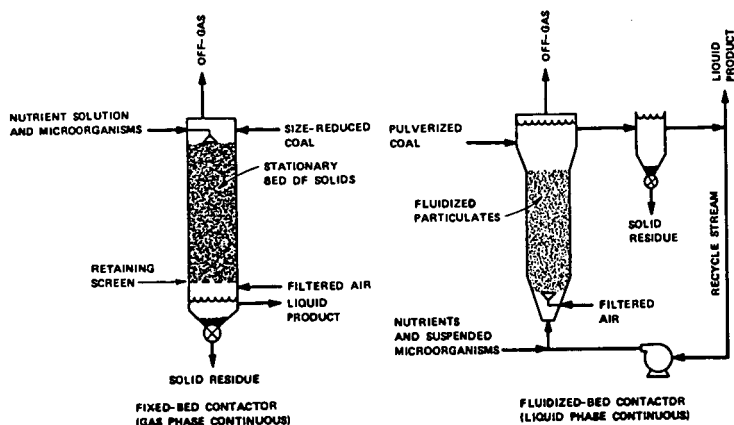
Figure 1

ORNL DWG 85-1243

Fig. 1 Two possible bioreactor configurations for the microbial liquefaction of coal.

Figure 2

ORNL DWG 88-9890
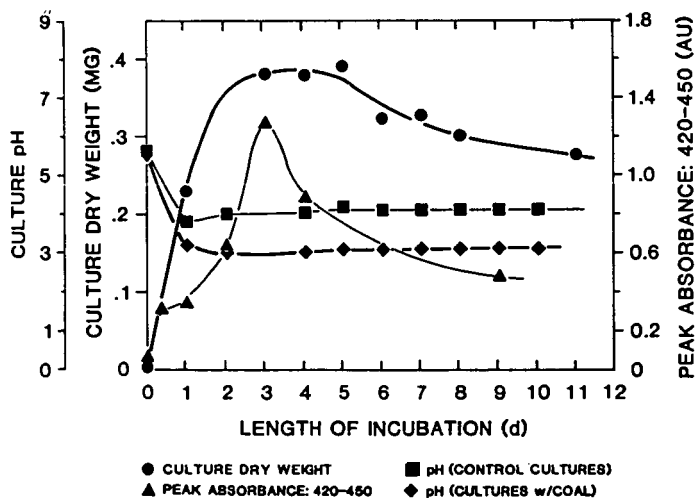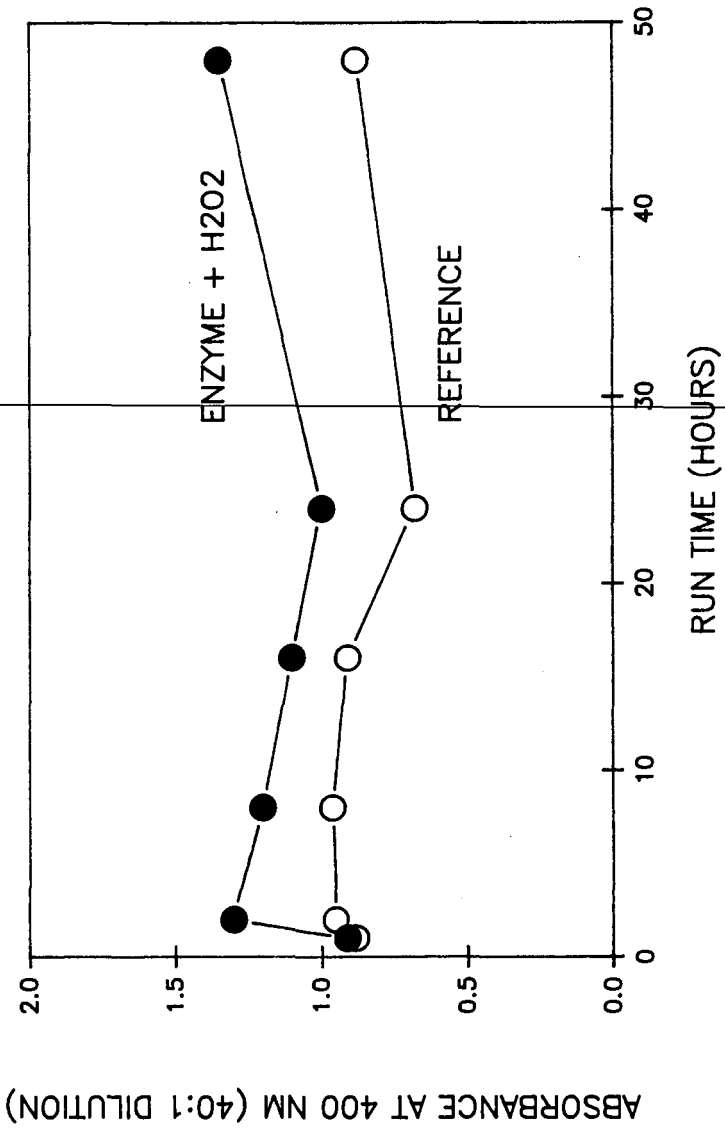
Fig. 2 Coal solubilization in an acidogenic system.

FIGURE 3

DISSOLUTION OF PREOXIDIZED SUBBITUMINOUS COAL
IN HYDROUS DIOXANE IN A FLUIDIZED BED

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MICROBIAL CONVERSION OF COALS TO CLEAN FUEL FORMS

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ABSTRACT

Anaerobic cultures have been used for the production of methane and alcohols from coal. Cultures were adapted from natural inocula collected from sources such as sewage sludge and horse manure. A 1% (w/v) slurry of leonardite, lignite, or subbituminous coal was used in the incubations. Methane was produced from all cultures, including some untreated coals, to a greater extent than in control cultures. Over several months of adaptation, methane production capacity increased considerably. Volatile fatty acids (VFAs) were identified as intermediates in the conversion of coal to methane. A proposed scheme for the conversion is breakdown of the coal polymer by a series of organisms and metabolism of the fragments to methane precursors such as VFAs. A mixture of short chain alcohols was produced by cultures grown in the presence of methane inhibitors. These cultures after prolonged adaptation show potential for use in larger scale bioreactors for the production of gaseous and liquid fuels.

INTRODUCTION

Recent interest in the use of microorganisms to process coal has led to the investigation of sulfur removal from coal as well as the conversion of coal to water soluble forms. Prior work in this laboratory has focused on investigating the mechanisms of formation of these water soluble compounds from coal by microbial as well as chemical means and characterization of the resulting products (ARCTECH, Inc. 1988a). The nonvolatile nature of these coal products inhibits their use as a clean fuel even though a majority of the ash and a proportion of the sulfur has been removed. In the current work, we have investigated the use of these coal products as well as untreated coals for the production of gaseous and liquid fuels (ARCHTECH, Inc. 1988b).

EXPERIMENTAL PROCEDURE

Coals--Both untreated coals and coal depolymerization products were used as substrates (1% w/v and 1% v/v, respectively) for methane, alcohol, and acid production. Two lignites were used as raw coal substrates. For the depolymerization products, three coals, North Dakota lignite, Beulah lignite, and Wyodak subbituminous, were treated with chemical or biological agents to yield water soluble products of a range of molecular weights.

<u>Anaerobic Cultures</u>--Anaerobic samples were collected from environmental sources and inoculated into an anaerobic medium containing coal or coal products. The procedures of Hungate, as modified by Bryant (1972) and by Balch and Wolfe (1976) were followed throughout. Serum stoppered tubes or serum bottles capped with black butyl rubber stoppers and crimp sealed were used for incubations.

Enrichment cultures were developed through successive transfers from each inoculum to fresh medium containing coal or coal depolymerization products every 28-45 days over a period of several months. Slow cultures were adapted to the coal or coal products over longer periods of time. Enrichment cultures were also developed in the presence of the methane inhibitors, 2-bromoethanesulfonic acid (BESA) and monensin, to allow the accumulation of acids and/or alcohols.

<u>Chemical Analysis</u>--All anaerobic fermentation products were quantified using gas chromatography. For gas analysis, a 10' x 1/8" O.D. stainless steel column packed with 100/120 mesh Carbosieve S-II was used. Methane, carbon dioxide, nitrogen, and hydrogen were determined using a thermal conductivity detector. The oven was programmed to hold at 100^oC for 1 minute then to increase temperature at a rate of 20^oC per minute to 200^oC. Total gas production was determined by syringe displacement and was used to calculate mole % of methane in the gas phase.

Alcohol analyses were performed with either a 6' x 2 mm I.D. (1/4" O.D.) glass column packed with 1% SP-1000 on 60/80 mesh Carbopack B (first 3-4 inches 10% SP-1000 on 100/120 mesh Supelcoport) or a 2.4 M x 2 mm I.D. (1/4" O.D.) glass column packed with 0.3% Carbowax 20M on 80/100 mesh Carbopack C using a flame ionization detector. The oven was programmed from 60^oC to 150^oC at 20^oC per minute then to 190^oC at 5^oC per minute and held. Analysis was performed by direct aqueous injection after centrifugation of samples to remove particulate matter.

Volatile fatty acids were primarily analyzed with an 8' x 2 mm I.D. glass column packed with 4% Carbowax 20M on 80/120 mesh Carbopack B-DA using a flame ionization detector. The oven was programmed to hold at 100^oC for 1 minute then increase temperature to 180^oC at 15^oC per minute then to 205^oC at 10^oC per minute. Samples were diluted 1:1 with 0.06 M oxalic acid and centrifuged to remove particulates before injection.

RESULTS

<u>Production of Methane From Coal and Coal Products</u>

Figure 1 presents the time course of methane production from an untreated North Dakota coal and from Beulah lignite coal products. Methane production from samples containing coal or coal depolymerization products was higher than that of control cultures, demonstrating the conversion of coal carbon to methane. Similar results were obtained with other natural inocula, although not all cultures produced methane from every coal. A shift in the color of the reaction medium was observed during the course of incubation that was associated

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with depolymerizing activity. The flattening out of the production curve indicates depletion of nutrients in the culture.

The effect of biological or chemical depolymerization of the coal on methane production was investigated with several different cultures. Data in Table 1 demonstrate increased production of methane in samples containing biologically produced coal depolymerization products. Methane is also produced from chemically depolymerized substrates although at a lower level. These experiments indicate that the products formed during biological and chemical depolymerization differ.

Identification of Alcohols and Acids in the Culture Medium

Our experiments indicated that methanogens were present and active in the adapted cultures. Methanogens are known to convert only simple organic molecules such as acetate and carbon dioxide to methane. Further experiments were initiated to determine the existence of methane precursors in the medium. To accumulate these precursors, methane inhibitors were added to enrichment cultures. Monensin serves as an inhibitor of specific enzymatic steps of methane production, whereas 2-bromoethane sulfonic acid (BESA) is an analogue of coenzyme-M which is required for methane production.

In almost all of the samples, BESA completely inhibited the production of methane. Monensin, however, inhibited methane production only from a single culture adapted to Beulah coal products. Results of analyses of culture media during methane inhibition studies are reported in Tables 2 and 3. Several short chain alcohols, including methanol, ethanol, propanol, and butanol, were produced by these cultures from either coal or coal depolymerization products. Acetone was also produced in small quantities by some cultures. No alcohols were produced in the absence of coal or coal products.

Product ratios varied depending on the inhibitor added to the medium. Ethanol was clearly the major alcohol produced by all cultures, with concentrations ranging between 757 and 2288 ppm. Methanol accumulated up to 316 ppm only in the presence of monensin. Propanol and butanol formation was favored in the presence of BESA. Total alcohol production decreased as the pH of the medium increased with no production of alcohols at pH 7.

In addition to alcohols, several short chain organic acids were detected in the culture medium (Table 3). Acetic acid was the principal organic acid detected, accumulating to a level of 1312 ppm. Small quantities of propionic, butyric, and valeric acid were also identified. Control samples without coal or coal depolymerization products produced minute quantities of acetic acid. No other organic acids were detected in these control samples.

DISCUSSION

The results reported in this paper demonstrate the feasibility of converting untreated lignite coals or depolymerized products of lignite and

subbituminous coals to methane by anaerobic cultures. Methane is produced from enrichment cultures in excess of that produced in the absence of coal or coal products. The ability of the cultures to produce methane increased as the enrichment period increased, demonstrating bacterial adaptation to use of coal or coal depolymerization products. The next step in culture development will be transfer of the most promising culture to a small bench-scale bioreactor to monitor continuous production of methane from coal or coal products.

Enrichment cultures are also capable of converting coal and coal-derived material to short chain alcohols and acids when methane production is inhibited. Although total alcohol concentration in the medium is less than one percent, the amount of alcohol produced by the cultures has steadily increased as the cultures adapted to the new substrates. Time course experiments are in progress to determine the production of alcohols and acids over an extended period.

Anaerobic enrichment cultures are capable of converting both untreated lignite coal and depolymerized coal products to methane, alcohols, and volatile fatty acids. A distinct preference for biologically derived coal products rather than chemically derived products was observed. Alterations in the coal molecule effected by microorganisms might be more conducive to further microbial attack than chemical modifications.

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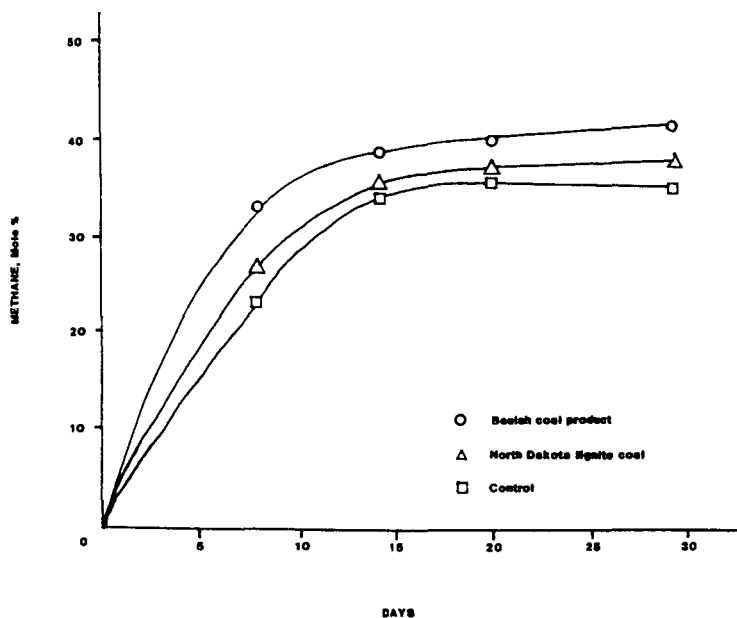
Figure 1. Production of methane from a ND Coal and Beulah Coal Product
by Natural Inocula

Table 1. Direct Production of Methane from Coals by
Anaerobic Bacteria

	Methane Produced,** Mole %	
Coals	2 Mos. Adaptation	6 Mos. Adaptation
Leonardite	7.8	25.6
	11.4	19.3
Texas lignite	9.1	----
	6.4	----

* 1% (w/v) coals were used.
** Methane from control samples (no coal) was substracted.

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Table 2. Production of Alcohols from Untreated Leonardite Coal and
Depolymerized Products of a Beulah Coal Using Anaerobic
Microorganisms

			Alcohols Produced, ppm			
pH	Coal	Inhibitors	Methanol	Ethanol	Propanol	Butanol
5.0	L	BESA	7.2	2288.2	22.3	18.6
5.0	L	Monensin	275.3	2053.3	3.9	1.9
5.0	L	Control	0	10.9	25.6	0
6.0	L	BESA	7.06	1335.9	173.5	38.3
6.0	L	Monensin	315.9	1975.5	1.7	0
6.0	L	Control	0	16.2	19.9	1.7
6.0	B	BESA	13.5	756.5	202.9	88.9
6.0	B	Monensin	129.2	911.4	10.1	8.5
6.0	B	Control	29.1	4.9	4.5	6.2

Table 3. Production of Short Chain Acids from Coals and Depolymerized
Coal Products Using Anaerobic Microorganisms

		Acids Produced, ppm			
Coal	Inhibitors	Acetic	Propionic	Butyric	Valeric
L	BESA	925.8	70.9	28.8	280.7
L	Monensin	626.5	77.7	34.1	266.1
L	Control	34.7	0	0	0
L	BESA	1311.5	306.5	80.7	501.0
L	Monensin	618.9	92.6	83.8	436.5
L	Control	4.5	0	0	0
B	BESA	932.4	233.9	62.5	0
B	Monensin	805.0	261.8	57.9	0
B	Control	99.6	118.7	0	0
TXL	BESA	885.7	158.4	34.7	0

L   - Leonardite
B   - Beulah lignite
TXL - Texas lignite
BESA - 2-bromoethanesulfonic acid

MICROBIAL OXIDATION AND REDUCTION OF SULFUR IN COALS FOR SPECIATION AND DESULFURIZATION

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1. INTRODUCTION

The microbial removal of sulfur from coal by oxidative means is well documented, especially with regard to pyritic sulfur removal by organisms such as *Thiobacillus ferooxidans* and *Sulfolobus acidocaldarius*. There also have been reports of the removal of organic sulfur from coals, (1,2) although generally less success has been achieved along these lines. Two aspects of microbial coal interaction which have received less attention are the use of microbes to elucidate coal sulfur chemistry and the removal of coal sulfur by bio-reduction. It is unlikely that there are significant amounts of elemental sulfur in pristine coal samples (3) but the recent work of Narayan suggests the presence of elemental and/or polysulfide sulfur in some weathered coals (4). By ASTM techniques these sulfur forms would be counted as "organic" sulfur. The lack of a direct assay for organic sulfur makes it difficult to establish a clear connection between microbial metabolism and the cleaving of covalently bound sulfur from the coal matrix. It also ignores any chemical interconversion of sulfur species which may occur during weathering, desulfurization, or analytical processing.

In this study we have made use of organisms which selectively metabolize elemental sulfur, either oxidizing it to sulfate at 28°C (*Thiobacillus thiooxidans*) or reducing it to hydrogen sulfide at 98°C (*Pyrodictium brockii* and *Pyrococcus furiosus*). We have pursued both the development of a bioassay for the speciation of sulfur in coals and novel desulfurization methods. The quantification of elemental sulfur by bioassay was confirmed with CS_2 extraction of the coals. Narayan has suggested that a significant fraction of so called organic sulfur in coals may be amenable to transformation to free elemental sulfur under mildly oxidative conditions. We will attempt to oxidize several coals using such techniques to investigate the possibilities for novel routes to microbial "organic" sulfur removal. The activity of the organisms toward polysulfide sulfurs (amorphous), partially oxidized species, and other model compounds (i.e. dibenzothiophene) will also be investigated.

The reduction of sulfur to hydrogen sulfide may avoid one of the problems involved with oxidative coal desulfurization, namely, the re-deposition of insoluble sulfate bearing minerals such as jarosite from the leachate when sulfate concentrations become too high. We are also investigating the activity of cell free extracts and lysed membrane preparations towards coal desulfurization.

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1.1. The Microorganisms

Thiobacillus thiooxidans is an acidophilic, aerobic, sulfur-oxidizing chemolitho-trophic bacterium. Strain 8085 was obtained from the American Type Culture Collection, Rockville, MD. *Pyrodictium brockii*, an autotroph, and *Pyrococcus furiosus*, a hetero-troph, are both hyperthermophilic, anaerobic, sulfur-reducing archaebacteria, capable of growth above 100°C (5,6,7). *P. brockii* strain DSM 2708 and *P. furiosus* strain DSM 3638 were both obtained from the Deutsche Sammlung von Mikroorganismen, Gottingen, Federal Republic of Germany.

2. EXPERIMENTAL METHODS

2.1. Coals .

Samples of four different coals were used in this study. Indiana no. 5 (IBCSP sample no. 6) and Illinois no. 6 (IBCSP sample no. 1) coals were obtained from C.W. Cruse, Direc-tor of the Illinois Basin Coal Sample Program. Victorian brown coal (Australia) was obtained from D.J. Allardice, Coal Corp. of Victoria, through L. Atherton of the Electric Power Research Institute. A waste coal from Indiana (bog coal) was obtained from R. Narayan, Purdue University.

2.2. Coal Biodesulfurization Experiments

2.2.1. Thiobacillus thiooxidans. Coals to be used in these experiments (200-400 mesh) were sterilized by heating in a vacuum oven at 85°C for one hour on each of three succes-sive days. Coal (0.5 to 1.5 g) was aseptically added to 50 ml of the sterile low sulfate medium (minus elemental sulfur) in 250 ml conical flasks. The flasks were inoculated with an active culture of *T. thiooxidans* previously grown in the low sulfate medium containing elemental sulfur. Coals were tested in duplicate or triplicate flasks. Sterile controls and flasks containing cells and no coal were run with each experiment. Flasks were incubated at 28°C with shaking at 200 r.p.m. After sulfate concentrations leveled off (generally after one to two weeks), the difference in solution sulfate concentrations between inoculated and uninoculated flasks was determined. From this value the amount of elemental sulfur converted to sulfate was calculated.

2.3.2. Pyrodictium brockii and Pyrococcus furiosus. Coals to be tested (200-400 mesh) were washed with reverse osmosis water and then dried in an air oven at 70°C for about four hours. Coals were tested in the range of 0.2 to 2.0 % pulp density (w/v). Bottles were incubated quiescently at 98°C. Gas phase H_2S was assayed by syringe injections to a Varian 3700 gas chromatograph using a thermal conductivity detector, a 6-foot, packed Hayesep-N column, and a carrier gas flow rate of 30 cc/min (helium). The total amount of sulfide generated was estimated using Henry's Law. Cell densities were determined by direct count of periodically taken subsamples. The cells were stained with acridine orange and visualized on 0.2 μm filters using epifluorescence microscopy.

2.3. Chemical Determinations

Sulfate ion in solution was determined turbidimetrically using barium chloride pre-cipitation (8). Total sulfur content in coals was determined using a modified high tem-perature method (9) employing a Leco (St. Joseph MI) furnace and automatic titrator. The method is based on an ASTM method for sulfur in petroleum products (10). NBS coal standard reference materials were used for system calibration. Forms of sulfur in coals (sulfatic, pyritic, organic) were determined using ASTM procedures (11). Elemental sulfur was determined chemically by extracting 0.5 g of coal with 25 ml of CS_2 at room tempera-ture for at least 12 hr. The coal was removed by filtration and the CS_2 was evaporated to dryness. The residue was taken up in hexane and assayed for sulfur colorimetrically (12).

3. RESULTS

3.1. Sulfur Oxidation by *Thiobacillus thiooxidans*

The amount of sulfur in various forms in the test coals is shown in Table 1. Especially noteworthy is the very low level of pyritic sulfur in the Australian coal and the very high level of total and sulfatic sulfur in the bog coal. This latter coal is evidently very weathered.

Small amounts of sulfate above control values were produced when *T. thiooxidans* was incubated with Indiana and Illinois coal samples indicating low levels of elemental sulfur in these coals. No significant differences in sulfate concentrations were found between inoculated and uninoculated flasks containing the Australian brown coal. However, significant sulfate levels above control values were seen with the Indiana bog coal. Calculations of the elemental sulfur content in these coals based on sulfate production are shown in Table 2. Very little elemental sulfur was detected by bioassay in the first three coals, corresponding with the results of CS_2 extraction. About 1.2% (weight) sulfur as elemental sulfur was detected by bioassay in the bog coal. The total sulfur content in the bioprocessed bog coal was about 1% lower than sterile controls. This corresponded closely to the loss in "organic sulfur" from the coal (Table 2). By ASTM definitions, the organisms removed about 25% of the "organic sulfur" from the coal.

3.2. Sulfur Reduction by *Pyrodictium brockii*

Table 3 shows estimates of elemental sulfur content of the bog coal by bioassay. Liquid phase sulfide concentrations were estimated assuming the system was at equilibrium at the end of the experiment and using Henry's law to calculate dissolved sulfide concentrations. Since the pH of the sample was typically about 5.5, it was assumed that all dissolved sulfide was molecular hydrogen sulfide. The inoculated samples containing the bog coal typically yielded gas phase sulfide concentrations 4 to 5 times those obtained from uninoculated controls and inoculated samples containing no coal. Samples of other coals used in this study showed no biotic sulfide generation although bacteria could be grown in these samples if spiked with elemental sulfur (data not shown). It is interesting to note that the drop in ASTM organic sulfur levels of about 30-40% correspond well with the elemental sulfur estimate by bioassay.

3.3. Sulfur Reduction by *Pyrococcus furiosus*

Table 4 shows the activity of *P. furiosus* on the coal samples used in this study. Note that for pulp densities of 0.5% (weight), growth was strongest for the bog coal. In fact, significant amounts of sulfide production were noted only in that sample.

As can be seen, the level of sulfide production and cell yields are proportional to the amount of coal added to the medium. The three replicates at the 2% (weight) pulp density show that reproducibility in these experiments is good. The uninoculated with coal and inoculated without coal control bottles had only trace amounts of H_2S. These results strongly suggest that coal sulfur was used as a substrate by the cells.

Not reported are bioassay estimates of elemental sulfur in the bog coal. Unlike *P. brockii* which grows at a pH of 5.5, *P. furiosus* grows at a pH of about 7.0. Because sulfide levels in the liquid phase were not measured directly, estimates of dissolved sulfide levels at pH 7 or higher are more difficult to make. The liquid phase dissociation of molecular hydrogen sulfide must be accounted for. Nonetheless, initial estimates indicated that elemental sulfur in the bog coal based on sulfur reduction by *P. furiosus* was between 1% and 2% by weight. Direct solution sulfide analysis will improve these estimates.

4. DISCUSSION

Several reports of microbial removal of organic sulfur from coal have appeared in the literature. However, the definition of organic sulfur is not precise or universal. By ASTM methods, organic sulfur represents all sulfur that is not pyritic or sulfatic. If significant elemental sulfur occurs in coal or is produced during studies on coal biodesulfurization, the apparent success of organic sulfur removal will depend on the analytical scheme. By ASTM designations all three organisms used in this study removed significant (25-40%) amounts of the "organic" sulfur present in the bog coal. At the same time, in the other three coals, very little elemental sulfur was detected by bioassay or CS_2 extraction. In these coals, a loss of ASTM organic sulfur following bioprocessing (which we did not detect) would be interpreted as a loss of true organic (C-S) sulfur. It is important, therefore, that investigators determine if elemental sulfur is present in their test coals, or perhaps more important, if it forms during experimentation as a result of biotic and/or abiotic reactions.

A more complicated situation arises in consideration of data presented by Narayan et al. (4) suggesting the presence of polysulfides in coal which oxidize on weathering to produce free elemental sulfur. We have not yet determined with model compounds whether or not it is likely that *T. thiooxidans*, *P. brockii* or *P. furiosus* (or other microorganisms) can attack such polysulfide linkages directly. Experiments are needed to determine if oxidative treatment of coals leads to the production of elemental sulfur and if polysulfides are attacked by these or other microorganisms. If such forms of sulfur are important in coals, there are very good prospects for their removal. However, the organisms which would remove this type of organic sulfur will be different from the organisms which would remove, for example, thiophenic sulfur.

Ultimately, definitions do not matter if the sulfur can be removed from coal. However, the choice of organisms and engineering designs will be quite different depending on the forms of sulfur that the organisms and their enzymes encounter.

5. ACKNOWLEDGMENTS

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Table 1. Sulfur in Test Coals by ASTM Procedures (%)

	Total S	Sulfate S	Pyritic S	Organic S
Illinois No. 6	4.25	0.08	0.73	3.44
Indiana No. 5	3.91	0.12	1.57	2.22
Australian	4.35	0.63	0.03	3.69
Bog Coal	11.6	5.20	2.32	4.06

Organic S = Total S - Sulfate S - Pyritic S
(ASTM Method 5.05)

Table 2. Elemental Sulfur in Coals - Thiobacillus thiooxidans

Coal Type	Total S (%)	S° (%) (bioassay)	Total Sulfur Determinations (%)		
			After CS_2 extraction	After Thiobacillus thiooxidans	sterile
Ill. #6	4.25	0.02	4.29	3.97	3.84
Ind. #5	3.91	0.03	3.76	3.39	3.49
Aust.	4.35	trace	4.50	nt	nt
Ind. Bog	11.6	1.19	8.37	5.26	6.22

nt - not tested

Table 3. Pyrodictium brockii on Bog Coal

	Total S (wt %)	Sulfatic S (wt %)	Pyritic S (wt %)	Organic S (wt %)	S° (wt %) (bioassay)
sterile + cells (Low Inoc.)	5.97 4.22	0.09 0.14	1.82 1.81	4.06* 2.27 $\Delta = 1.79$	1.62
sterile + cells (High Inoc.)	5.97 4.98	0.09 0.15	1.82 1.96	4.06** 2.87 $\Delta = 1.19$	1.40

apparent organic sulfur removal: * 44.1% and ** 29.3% respectively

Table 4. Activity of Pyrococcus furiosus on Coal Samples

Coal Type	Pulp Density	Cell Growth	Gas phase H_2S Production (μmoles)
Illinois No. 6	5.0	+	trace
Indiana No. 5	5.0	+	trace
Australian	5.0	+	trace
	0.0	+	trace
Bog Coal	5.0	+	43.2
Bog Coal	10.0	++	72.4
Bog Coal	20.0	uninoc.	trace
Bog Coal	20.0	+++	104.4
Bog Coal	20.0	+++	100.8
Bog Coal	20.0	+++	108.9

Microbial Desulfurization of Dibenzothiophene

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Abstract

Concerning the sulfur removal from coal before combustion
there is considerable interest in microbial methods as
pyrite oxidation and elimination of organically bound sulfur
from coal. Using organic sulfur compounds relevant for coal
the mechanism of desulfurization was investigated. We
isolated a defined mixed culture (FODO) capable to utilize
dibenzothiophene as sole sulfur source for growth, while
benzoate was used as carbon source. The mixed culture FODO
consists of an *Alcaligenes denitrificans* subspecies and a
Brevibacterium species. Two metabolites of the degradation
and dibenzothiophene-5-dioxide. The subsequent degradation
of dibenzothiophene-5-dioxide used as sole sulfur source
results in a release of sulfate ions into the medium. The
results suggest a sulfur specific oxidative mechanism for
removal of sulfur from dibenzothiophene.

Introduction

Coal combustion causes emissions such as dust, NO_x and SO_2,
which, if not diminished, are of environmental concern. To
prevent SO_2 emissions sulfur removal from coal before
combustion can partly be achieved by mechanical and chemical
processes (1). However, for future application microbial
methods are judged to represent a possible tool for sulfur
removal from coal (2;3). Coal contains, apart from pyritic
sulfur, traces of sulfates nd elementary sulfur, a
considerable amount of organically bound sulfur. This so-
called organic sulfur is an element of the chemical coal
structure integrated as thiol, disulfide, sulfide or
thiophene in the macromolecular coal matrix (4;5). Also the
occurrence of sulfur organo metallic species is established
for high sulfur coals (6). For the removal of organic
sulfur by means of biotechnological methods covalent C-S
bonds have to be cleaved. In consideration of the
inhomogenous structure of the coal, the enzymes involved
should therefore be specific for the sulfur of different
sulfur bonding types (regiospecificity). Experience is
still limited, concerning the metabolic pathway involved in
the removal of reduced sulfur from coal relevant model
compounds as dibenzothiophene (DBT). Early reports
suggested, that in bacterial DBT degradation the thiophene
nucleus remained untouched (9). However sulfate release by
an oxidative bacterial attack on DBT is reported by Isbister
and Doyle (7) and Kargi and Robinson (8).

This work contributes to the oxidative bacterial desulfurization of DBT, using DBT as sole sulfur source for bacterial growth.

Material and Methods

Chemicals

Dibenzothiophene (DBT) was purchased from Merck, Hohenbrunn, FRG; dibenzothiophene sulfone (DBT-5-dioxide), benzyl methyl sulfide, dibenzyldisulfide, dibenzylsulfinde, diphenylsulfide and poly (phenylene sulfide) from Aldrich-Chemie, Steinheim, FRG. Dibenzothiophene-5-oxide (DBT-5-oxide) was prepared as described by Gilman and Esmay (10). The product of the synthesis was contaminated with DBT and DBT-5-dioxide. Both of which were removed by high pressure liquid chromatography (HPLC). The identity of DBT-5-oxide was confirmed by GC-MS, the purity (98.3% DBT-5-oxide contaminated with 1.7% DBT-5-dioxide) by HPLC and GC. All other chemicals were obtained at the highest purity from commercial sources.

Apparatus and Analytical Methods

DBT was quantitatively removed from bacterial culture fluid by extracting with CH_2Cl_2 using equal volumes. DBT quantification was carried out by gaschromatography (Varian, mod. 3400, Darmstadt, FRG) using a Chrompack Sil 5 capillary column (25mX0.22mm) and a flame ionization detector with the following oven temperature program: $80^{\circ}C$ for 5 min, $10^{\circ}C$/min to $300^{\circ}C$ which was held for 5 min.

For metabolite characterization, samples of culture fluid were prepared by centrifugation at 10,000 g for 15 minutes to remove particles and cells. Metabolites of DBT degradation in the supernatant were detected at 220 nm or 290 nm after high pressure liquid chromatography (HPLC) using following equipment: Liquid chromatograph 655A-11 with autosampler (Merck, Darmstadt, FRG) and variable wavelength monitor (Knauer, Bad Homburg, FRG); columns (Knauer): length 250 mm, internal diameter 4 mm or 16 mm filled with LiChrosorb RP8 particles (Merck) of 5 um in diameter; solvent system: 50% acetonitril (v/v), 50% water (v/v) containing 0.12% H_2PO_4 (v/v).

Cochromatography was carried out with synthesized DBT-5-oxide and authentic DBT-5-dioxide at approx. the same concentrations as the metabolites.

For identification of metabolites the UV-spectra (spectrophotometer: Shimadzu UV-210 A, Langenfeld, FRG) were compared with authentic DBT-5-dioxide.

GC-MS analyses were performed with a double focussing mass spectrometer (Finnigan MAT-8200, EI 70 eV) and an INCOS datasystem. The determination of sulfate ion concentration in the culture fluid was routinely carried out by the method of Cypionka and Pfennig (11). Additionally sulfate and sulfite ion concentration are detected by ion chromatography (Dionex 2000-I). Growth of the cultures was monitored either spectrophotometrically by measuring the optical density at 578 nm with an Eppendorf photometer 1101 M (Eppendorf, Hamburg, FRG) or as protein using the Lowry method (12).

Media

Sulfur limitation required scrupulously cleaned glassware to prevent bacterial growth on contaminant sulfur. The medium consisted of a salt solution containing, per liter, 2.19 g NH_4Cl, 0.27 g KH_2PO_4, 0.2 g $MgCl_2$ x 6 H_2O, 0.1 g $Ca(NO_3)_2$ x 4 H_2O, 10 mg $FeCl_3$ x 6 H_2O and 1 ml of trace element solution. The medium was adjusted with 20 mM tris/HCl to pH 7.4 and was supplemented with 10 mM benzoate. The required amount of organic sulfur sources were put into culture tubes or flasks from stock solutions (DBT dissolved in diethyl ether, DBT-5-oxide/DBT-5-dioxide dissolved in CH_2Cl_2). AFter the organic solvents had been evaporated medium was added. The tightly closed culture tubes and flasks were sterilized at 121oC for 20 minutes. All organic sulfur sources used are stable as proved by GC and HPLC.

Enrichment and Isolation of Organisms

Enrichment of suitable microrganisms was achieved in a pneumatically operated percolator with external loop as described by Codner (13) containing 33% (w/w) sterilized postbog, 33% (w/w) raschig-rings and 33% (w/w) municipal sewage sludge as inoculum. To adapt the microbial population selectively to organic sulfur compounds, the percolator was supplemented with dibenzothiophene, benzyl methyl sulfide, dibenzyldisulfide, dibenzylsulfide, diphenylsulfide and poly(phenylene sulfide) at concentrations of the percolator fluid were incubated at 30°C on a rotary·shaker at 170 rpm in 10 ml screw-cap culture tubes (3 ml medium) with DBT (200 uM) as sole sulfur source and glucose (10 mM), glycerol (10 mM) or benzoate (10 mM) as carbon source. The isolation of organisms was carried out as described by Cook and Hutter (14), the vitamine soluation being omitted. Bacterial isolates were characterized according to their gram reaction, fatty acid composition and physiological parameters in part by DSM (Braunschweig, FRG).

2.5 Isolation of Metabolites

Cells were removed from the culture fluid by centrifugation (10,000 g, 15 min) and the supernatant was acidified to a final concentration of 0.1 M HCl and extracted with CH_2Cl_2 (1:1 v/v). After concentrating the organic phase to approximately 1% of the original volume the preparation was used for GC-MS analyses. Samples for isolation of metabolites by HPLC were transferred to the aqueous phase again.

Results and Discussion

Isolation and Characterization of Organisms

After simultaneous adaption of an enrichment culture to several organic sulfur compounds using a percolation equipment one stable bacterial mixed culture was obtained from percolator liquid. This culture was able to grow with DBT as sole sulfur source and benzoate as sole carbon source. Without sulfur source bacterial growth was strongly limited. Analogous enrichment procedures with glucose or glycerol as carbon source were not successful. After numerous isolation steps, a two species community (FODO) was obtained. The two organisms could be distinguished by the morphology of their colonies. On nutrient broth (NB) agar plates growth of organism FO (feeding organism) was always superior compared to organism (DO) degrading organism). Pure cultures of strain DO on NB agar plates formed very small and transparent colonies (diameter 0.5 - 1 mm, 30°C, 3 d), whereas colonies growing together with strain FO on the same plate are lightly yellow with a diameter of 4-5 mm (30°C, 3 d). This effect could be due to the need of diffusable nutrients or vitamines by strain DO.

Pure cultures of each strain were not able to grow with DBT as sole sulfur source.

Strain FO was identified as a non-motile subspecies of *Alcaligenes denitrificans* and strain DO as a non-motile *Brevibacterium* species.

Physiology of growth

The mixed culture FODO cultured with DBT (200 uM) as sole sulfur source and benzoate as carbon source grew with a doubling time of 8.5 h at the pH optimum of 7.4.

A molar growth yield of 9.8 kg protein per mol of sulfur was obtained with low concentrations (1-30 uM) of DBT or sulfate as sulfur source (Fig. 1). This indicates that the DBT-sulfur was as accessible to the culture as sulfate.

Comparable ranges of molar growth yields are described by
Cook and Hutter (14) for bacteria, isolated from sewage and
soil.

Table 1 showed that bacterial growth and DBT-degradation
remained almost constant, in spite of sulfate in varying
concentrations (0-100 uM) was present as a second sulfur
source. This indicates that at least the first step in DBT
degradation is not repressed by sulfate.

Catabolism of DBT

The bacterial mixed culture FODO was grown on DBT (200 uM)
with benzoate as carbon source. In the course of bacterial
growth DBT was degraded to a final concentration of 71 uM
concomitant to growth. In parallel sterile controls the DBT
concentration remained unchanged.

Furthermore the formation of two metabolites of DBT
degradation was observed (Fig. 2). The metabolite occurring
first was identified by GC-MS and cochromatography (HPLC) as
DBT-5-oxide. The second metabolite was identified by
cochromatography (HPLC) and UV-spectrometry as DBT-5-dioxide
(Fig. 3 and Tab. 2). DBT-5-oxide was produced concomitant
to bacterial growth up to a final concentration of 42 uM
after 80 h incubation. At the end of thelogarithmic growth
phase production of DBT-5-dioxide was quantified as a final
concentration of 18 uM (Fig. 2).

The yield of the products obtained, was about 46% of the
amount which would be achieved by totally conversion of DBT
to the concentration of about 15 uM sulfur is necessary for
an optimal growth of the cells. Several organisms are able
to accumulate sulfur intracellularly (15) but this seems not
to be true with the mixed culture FODO because DBT grown
cells, washed and incubated in sulfate free medium are
strongly limited in growth due to sulfur depletion.

The discrepancy in sulfur balance implies that at least one
other sulfur compound has to be accumulated during growth.
However no sulfate, sulfite or other additional metabolite
could be identified up to now.

In order to prove whether DBT-5-oxide and DBT-5-dioxide are
utilized by the bacterial culture as sulfur sources too,
growth experiments using this compound (25 uM) as sole
sulfur source with benzoate as carbon source were carried
out. As shown in Fig. 4 both the sulfoxide and the sulfone
were degraded in the course of cell growth, whereas the
growth of control cultures without any sulfur source was
negligible.

Using high concentrations of DBT-5-dioxide (2 mM) as sole
source of sulfur with benzoate as carbon source, in the

stationary phase of growth 21.2% (424 uM) of DBT-5-dioxide was degraded and a concentration of 200 uM sulfate, but no sulfite was detected in the supernatant culture fluid.   In sterile controls neither sulfate nor sulfite could be measured.   Considering the amount of 15 uM sulfur the bacterial cultures needed for optimal growth, these results confirm the aforementioned hypothesis, that at least one additional unknown sulfur compound accumulates.

Further investigations are directed to isolate this "missing sulfur compound" and the first sulfur free metabolite to examine the C-S bond cleavage occurring in the desulfurization of DBT.

The presented data indicate a sulfur specific attack on DBT by a bacterial two species community which utilizes DBT as sole source of sulfur.   We propose that sulfur elimination proceeds via the corresponding sulfoxide and sulfone (Fig. 5).

This is the first report of the bacterial degradation of DBT serving as sulfur source, though similar reactions have been reported: The microbial oxidation of thiophenes to the corresponding sulfoxides and sulfones is reported by Holland et al (16) and Fedorak et al (17), but no further degradation could be achieved.   Kaufmann and Kearney (18) reported on the degradation of a methylthio-S-triazine by soil bacteria.   The desulfurization of this organic sulfide proceeds through the sulfoxide and the sulfone whereby methyl sulfonic acid is suspected to be an intermediate (14). A *Pseudomonas* species is described to desulfurize DBT by cooxidation with benzoate by Isbister and Doyle (7).   Using DBT as carbon and energy source for growth of *Sulfolobus acidocaldarius* sulfate release was measured by kargi and Robinson (8).   However biochemical reactions of the aforementioned DBT degradation are not reported in detail, the initial attack may be of similar type as presented in this work.   This initial reaction occurring in DBT degradation (Fig. 5) support the approach to develop specific mechanisms for microbial removal of organically bound sulfur from coal.

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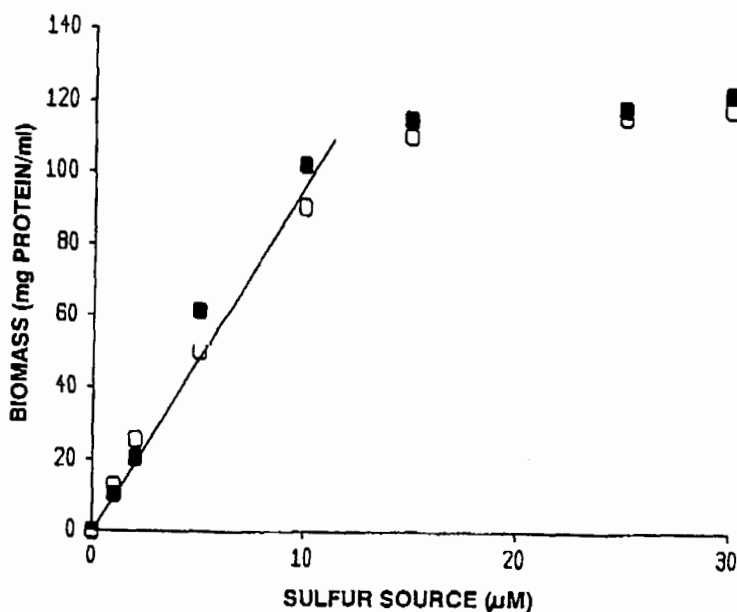
Figure 1: Biomass production at the stationary growth phase of the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. at different concentrations of sulfur sources. ◻ dibenzothiophene, ▆ sulfate

Figure 2: Growth of the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. with dibenzothiophene as sole sulfur source and benzoate as carbon source. ▆ optical density, ◻ optical density without sulfur source added, ● dibenzothiophene, △ dibenzothiophene-5-oxide, ▲ dibenzothiophene-5-dioxide.

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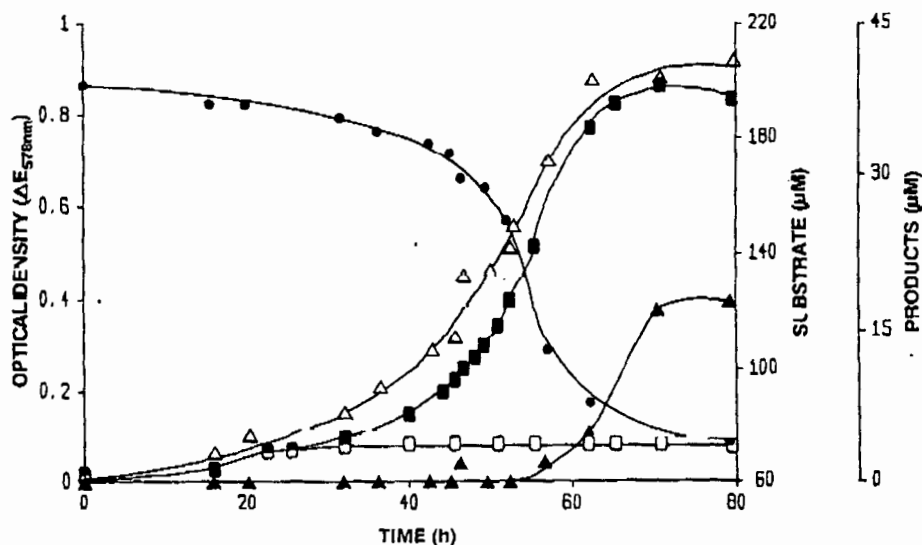
Figure 4: Growth of the mixed culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. with dibenzothiophene-5-oxide (a) and dibenzothiophene-5-dioxide (b) as sole sulfur source and benzoate as carbon source. ■ optical density, ☐ optical density without sulfur source added, △ dibenzothiophene-5-oxide, ▲ dibenzothiophene-5-dioxide.

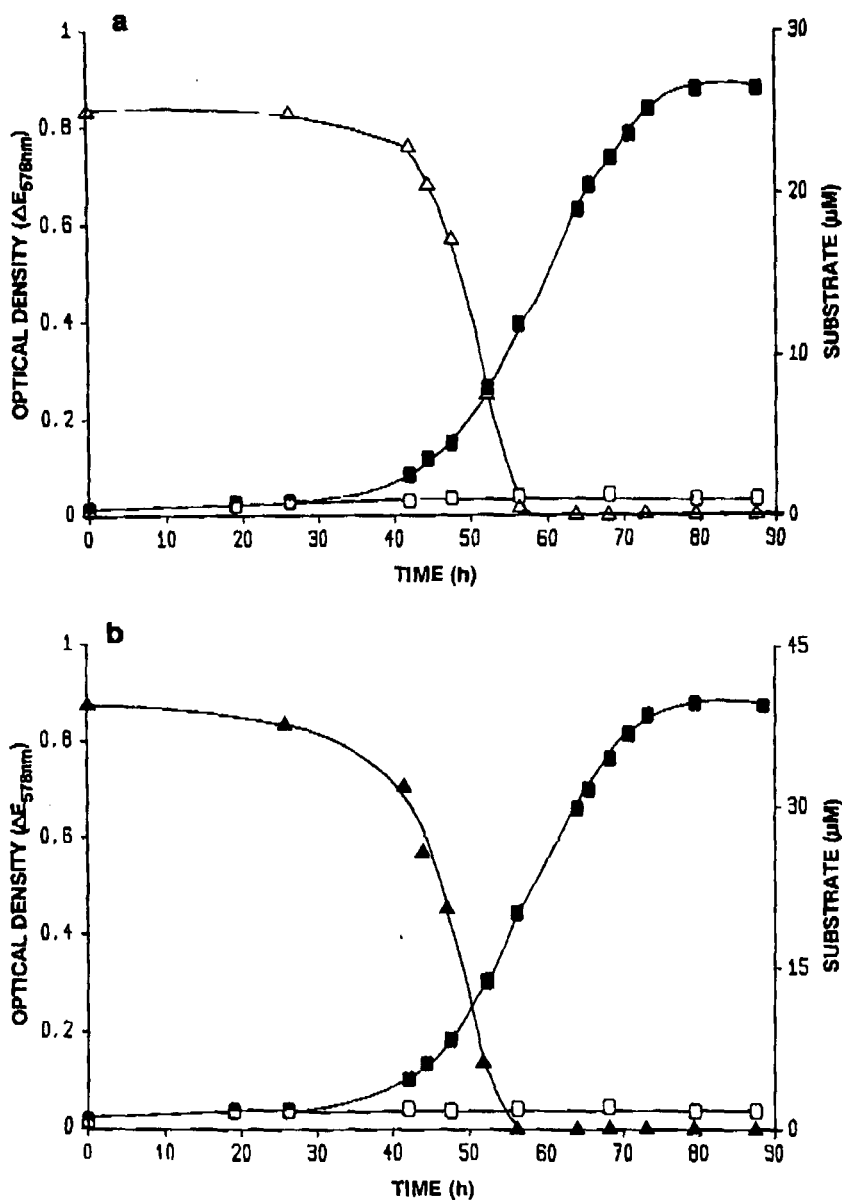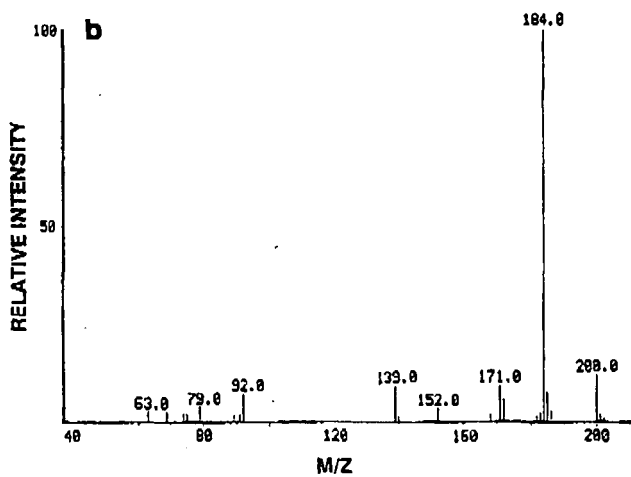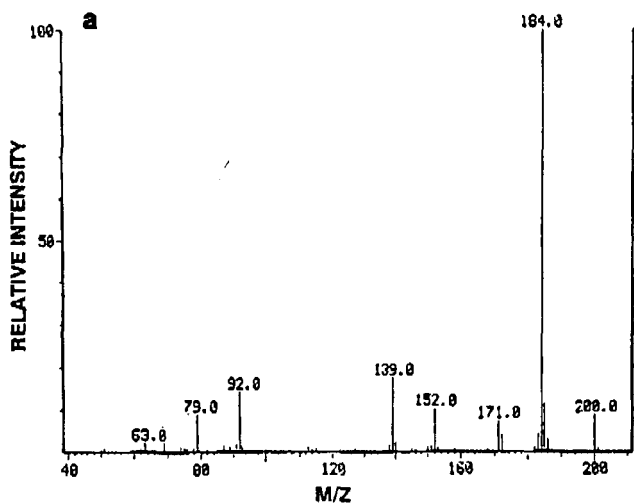Figure 3: Mass spectra of (a) dibenzothiohpene-5-oxide produced
from dibenzothiophene by the mixed culture of *Alcaligenes
denitrificans* and *Brevibacterium* sp. and (b) authentic
dibenzothiophene-5-oxide (library search).

DBT

DBT-5-oxide

DBT-5-dioxide

SO_4^{--}

X
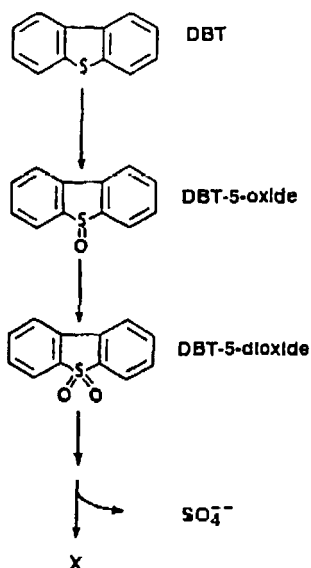
Figure 5:
Proposed initial reactions of
the desulfurization of dibenzo-
thiophene (DBT) by the mixed
culture of *Alcaligenes denitri-
ficans* and *Brevibacterium* sp..

Table 1: Utilization of dibenzothiophene (100 µM) by the mixed
culture of *Alcaligenes denitrificans* and *Brevibacterium* sp. with
benzoate as carbon source in presence of various amounts of
sulfate.

Sulfate (µM)	optical density (E_{578nm})	dibenzothiophene degradation (%)
0	0.96	85.5
2	1.01	84.5
5	1.02	80.9
10	0.92	90.0
15	1.01	79.5
30	0.96	84.5
50	0.99	91.8
100	1.00	89.6

Table 2: Identification of metabolites fron dibenzothiophene (DBT)
released during growth of mixed culture of *Alcaligcnes
denitrificans* and *Brevibacterium* sp.

Product	Cochromatography[*] retention time (min)	UV-spectrometry[**] maxima (nm)
DBT-5-oxide	5.1	not assayed
DBT-5-dioxide	7.1	236/244/280/292/322

[*] HPLC, retention times are identic with authentic chemicals
[**] Maxima are identic with authentic DBT-5-dioxide

COAL DESULFURIZATION THROUGH

REVERSE MICELLE BIOCATALYSIS PROCESS

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ABSTRACT

A novel bioprocess using micelle biocatalysis has been attempted to minimize several disadvantages of conventional microbial coal desulfurization scale-up processes. The reverse micelle biocatalysis process consists of a water-immiscible organic medium, a surfactant, an aqueous phase and sulfur-oxidizing bacteria or enzymes. This process has been successful for removing sulfur from bituminous coal (Illinois coal #5). The preliminary results showed that coal desulfurization through the use of cell-free enzyme extracts of Thiobacillus ferrooxidans ATCC 19859 was better than that of bacteria itself. The use of enzymes has shown potential for commercial coal desulfurization processes as well. This same process is being applied to the thermophillic bacteria Sulfolobus acidocaldarius ATCC 33909. The implications of these experimental results will be discussed, including a hypothetical mechanism using reverse micelle biocatalytical process for coal desulfurization

INTRODUCTION

Since coal utilization as an alternative energy resource is associated with SO_2 emission, causing acid rain and acid fog during stages in the combustion process, there has been a great interest in the desulfurization of coals.

Microbial desulfurization methods are given great amounts of attention because biological removal of sulfur compounds prior to combustion of coals requires low capital and operating costs. Several researchers have demonstrated the feasibility of sulfur removal from pulverized coals by sulfur-oxidizing bacteria. However, this bacterial desulfurization process has not yet been applied on

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a commercial scale. This is partically due to the fact that the reaction rate in the bacterial process in comparatively slow, and the microbial cells must be in physcial contact with pyrite to decompose it[1,2]. In order to overcome these barriers, a new desulfurization process is needed.

During the last decade, there has been a rapid growth of the application of biocatalysts (whole cell, purified enzymes and cell organelles) in non-aqueous media. Recent research has revealed that the reverse micellar system -- solvent/surfactant/water -- affords protein solubilization and enzymatic chemical reactions[3,4,5]. Of special interest is the reversed micelle solution (oil/nonionic surfactant/water) which may generate large swollen micelles (globules). The "water pools" of the reversed micelle created by large head groups of the non-ionic surfactants might be used as a "microreactor."

The objective of this work is directed not only to investigate catalytic oxidation of ferrous ion by biocatalysts (bacteria and cell-free enzyme extracts) entrapped in the water pools of the reversed micellar system, but also to provide feasibility of sulfur removal from coal. We will describe here our preliminary results on the iron oxidation via reverse micelle biocatalysis, and on bituminous coal desulfurization.

EXPERIMENTAL

Pure cultures, Thiobacillus ferrooxidans ATCC 19859 and Sulfolobus acidocaldarius ATCC 33909, were obtained from the American Type Culture Collections and routinely maintained[6,7]. Cells were harvested after they were four days old, suspended in distilled water (pH 2.6 with H_2SO_4), and allowed to stand in the refrigerator. The resulting supernatant fluid was carefully collected, brought to a volume of 30 ml with distilled water (pH 2.8), centrifuged and stored in the refrigerator. 10 ml of stored cell suspensions (0.02 g/ml, dry weight) were sonicated in glycine-SO_4 buffer solution (pH 2.8) for one hour so that cell-free enzyme extracts were prepared.

The reversed micellar system included 0.01% nonionic surfactants (Tween 80) in mineral oil (light) (wt/vol) and 1% water phase (vol/vol). The appropriate rates of blowing air in the reversed micellar system were 40 ml m^{-1} at room temperature.

To measure a change of concentration of ferrous ion in the reversed micelle, the ferrous oxidation measurement described by Tamura et al.[8] was adopted. A stable complex of Fe^{2+} having color density by 1,10-phenanthroline assay was determined at 510 nm with the spectrophotometer (Figure 1).

Table 1. Sulfur content of Illinois coal #5

	PYRITIC %	SULFATIC %	ORGANIC %	TOTAL %
SULFUR	1.82	0.03	2.50	4.35

For this study, an Illinois #5 coal sample was collected from the Burning Stat #4 mine in Illinois. This sample was reported to hold 4.35% total sulfur (Table 1). An Illinois coal #5 was crushed and ground to 100 mesh. The obtained coal powder was slowly poured into the mineral oil containing 0.01% non-ionic surfactant (wt/vol). Then, bacteria and respective media (pH 2.4) were added into the organic phase. The mixed multiphase solution was sonicated and vigorously agitated. After incubation, coal particles were collected by centrifuge and washed with hexane and hot water to remove possible oil associated with the coal particles. The washed coal samples were dried and the content of total sulfur was assessed.

RESULTS & DISCUSSION

The iron assay at 510 nm shows a significant decrease of Fe^{2+} ion complex in the reverse micellar system containing the cell-free enzyme extracts of T. ferrooxidans (Figure 2). The Fe^{2+} level was rapidly dropped during the initial one hour; this could be due to enzymatic compounds responsible for iron oxidation entrapped in the water core of the reversed micelle. Such compounds could be a copper protein, rusticyanin and a Fe (II)-oxidizing enzyme from T. ferrooxidans. The result that iron could be oxidized by reversed micelle biocatalyst would support a feasibility of this system for the coal desulfurization process.

Figure 3 shows the data of total sulfur removal by the reverse micelle system. It was observed that there was about 13% total sulfur loss in coals (control) washed with solvent hexane and hot water, when comparing them with raw Illinois coal. The reason for that could be dissolution of elemental sulfur present in coal by solvent and hot water. It was positively observed that sulfur was removed from coal by the sulfur-oxidizing bacteria in the reverse micelle system. Remarkably, more than 37% of the total sulfur removal was found in the coals treated during a 24 hour period with the cell-free enzyme extracts reverse micelle system. It should be pointed out that the desulfurization reaction time by the cell-free enzyme extracts was comparatively very short. Also, the rate of sulfur removal by the cell-free enzyme extracts of Thiobacillus ferrooxidans was much higher than that of the bacteria. We therefore intrepret the preliminary results as showing that mineral oil/non-ionic surfactant/water allow protein solubilization and catalytic reaction. Hence, a reverse micelle biocatalysis seems to be a promising coal desulferization process. Further studies are being conducted with the thermophilic bacteria S. acidocaldarius. The implications of these results will be addressed.

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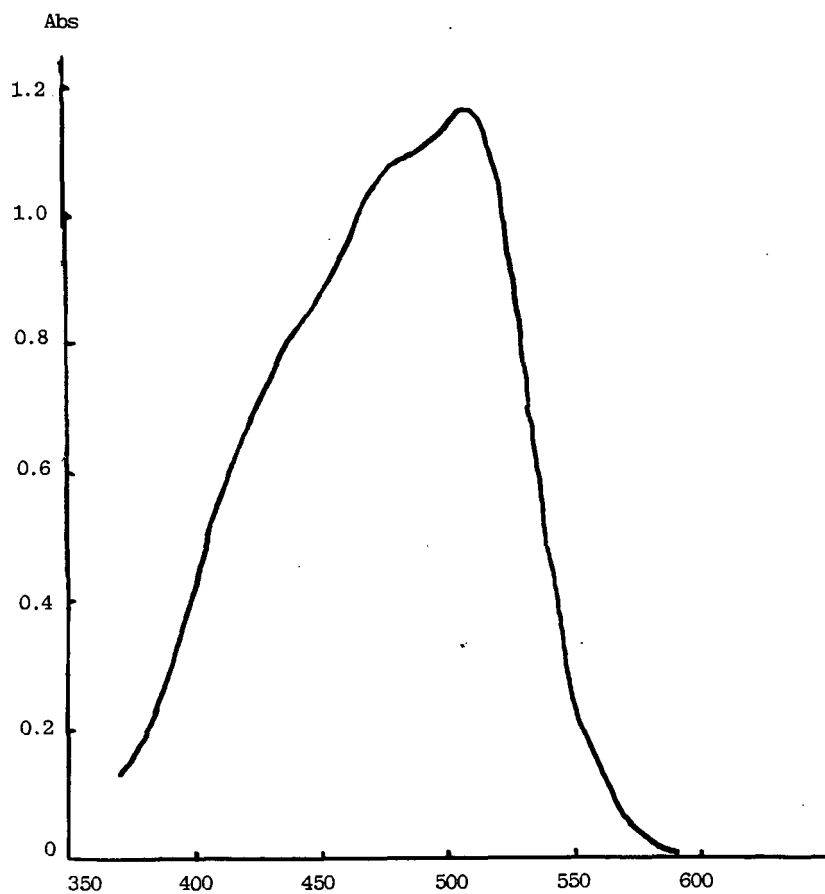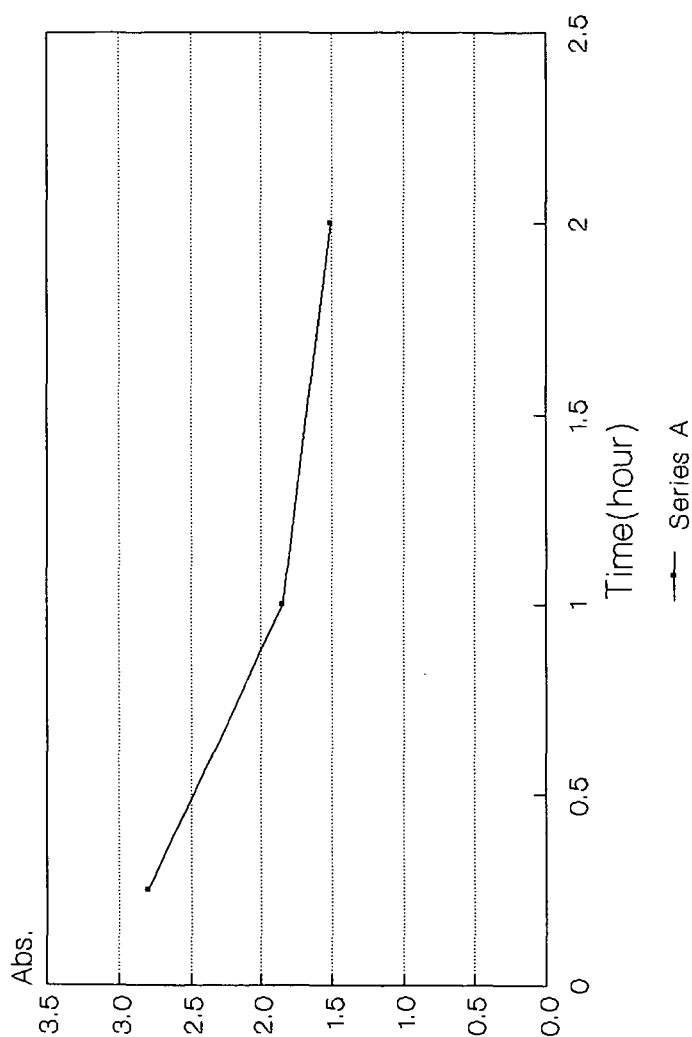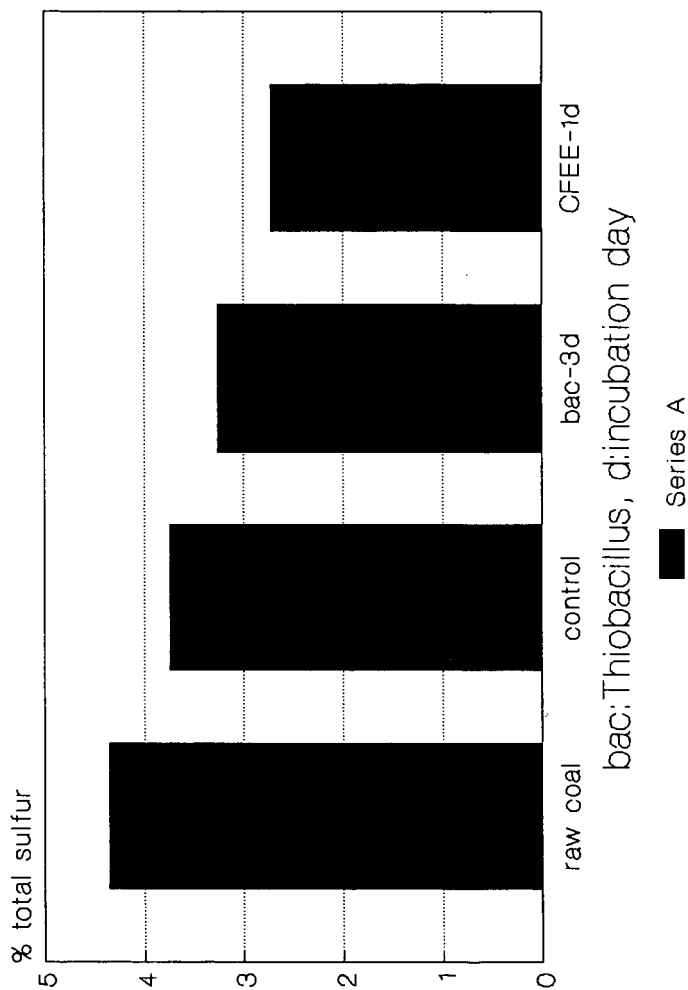
Figure 1. Absorption spectrum of 1,10-phenanthroline–iron complex extracted from reverse micelle system.

Figure 2. Iron oxidation via reverse micelle biocatalysts.

Abs.

Time(hour)

Series A

Cell-free enzyme extract of Thiobacillus

Figure 3. Coal desulfurization via reverse micelle biocatalysts.

bac:Thiobacillus, d:incubation day

Series A

CFEE:cell-free enzyme extract

EFFECTS OF MULTIVALENT CATIONS FOUND IN COAL
ON ALKALI- AND BIOSOLUBILITIES

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ABSTRACT

Extraction of low-rank coals with dilute hydrochloric acid resulted in the removal of a small percentage of total ash present. Calcium, and to a lesser extent, aluminum, magnesium and iron were present in acid extracts. Resulting coals were significantly more soluble in dilute alkali and more readily biosolubilized than water treated controls. These data suggest that some of the multivalent cations are present in coals as alkali insoluble complexes and contribute to the structural integrity of the coal.

INTRODUCTION

Since the observations of microbial coal solubilization in the early 1980's (1,2), there has been considerable interest in those mechanisms involved. Two mechanisms have been reported to be involved in coal biosolubilization: direct enzyme attack and production of alkaline materials. Enzymes implicated in the biosolubilization process include lacasses (3), ligninases (4), and peroxidases (4). Unfortunately, the degree of involvement of any of these enzymes in coal biosolubilization has yet to be rigorously demonstrated. Microbial production of alkaline. materials which are able to solubilize coals has been clearly demonstrated (5-8). These alkaline materials appear to be nitrogenous in nature and increase medium pH sufficiently to cause coal solubilization. This mechanism does not appear to alter coal molecular structure.

There is now suggestive evidence that a third mechanism is involved in coal biosolubilization. This mechanism involves the removal of multivalent metal ions resulting in coal becomming more soluble in dilute alkali. In this paper, we report how the removal of small amounts of ash present in coals dramatically increases coal alkali- and biosolubilization.

EXPERIMENTAL

Texas lignite was obtained from Northwestern Resources, Jewett, TX. Wyodak and Beulah Zap coals were from the Argonne Premium coal sample program, Argonne IL. Mississippi Wilcox lignite was obtained from a

weathered outcrop as previously described (9). Texas and Wilcox lignites were ground to -100 mesh and stored under a nitrogen atmosphere. Wyodak and Beulah Zap coals were recieved preground to -100 mesh and packaged under an inert atmosphere. While under a nitrogen atmosphere, coals were dried (50°C for 48 hours) and aliquots (1g) were extracted with either water or 6N HCl (50ml). Extractions were performed by shaking (140RPM) at ambient temperature (22±2°C). Coals were recovered by centrifugation (10,000xg for 10 min)and washed using deionized water until the pH of the wash water exceeded 3.0. Solubilities of coals in dilute alkali were determined by placing coal aliquots (1g) in 50mM Tris buffer (pH 8.0, 100ml), shaking (140RPM) for 72 hours and measuring the absorbance of the filtered fluid (Gelman GA-8 SUPOR, 0.2μM pore dia.) at indicated wavelengths.

Fungal strains RWL-40 (unidentified Basidiomycete) and YML-1 (Cunninghamella sp.) (9) were grown and maintained on yeast-malt agar slants at room temperature. Biosolubilization was tested by placing coal aliquots (0.5g) in late log cultures of each organism growing in yeast malt broth and monitoring the amounts of coal going into solution as a function of time. The amount of coal solubilized was estimated by measuring the absorbance of filtered culture fluids at indicated wavelengths.

Ultimate and proximate analyses were performed at the Fuel Characterization Laboratory, University of Utah, Salt Lake City, UT. Element analyses were performed on an ARL Model 3520 Atomic Absorption Spectrophotometer using the ICP mode.

RESULTS AND DISCUSSION

Preliminary drying at 50°C under nitrogen did not appear to oxidize coal samples, but did reduce coal moisture content to a consistent 3-6% (data not shown). Extractions of coals with 6N hydrochloric acid likewise did not result in significant increases in coal oxygen content as compared to water extracted controls (Table 1). These data indicate that no significant amounts of either acid catylized hydrolysis (e.g., ethers, esters, etc.) or oxidation occured. Acid extraction of coals reduced total ash content by a consistant 2-5% and appeared to be independent of original ash content. It is unlikely that any of the ash reduction is due to decreasing pyrite content because sulfur contents remain essentially unchanged.

Acid extracts of coals were a deep yellow or yellow-green color and contained a relatively large amount of calcium and lesser amounts of magnesium, aluminum, iron (Table 2). Sodium and potassium were also present. Very low concentrations of metals were observed in controls (water extracts). The total amount of metals observed in each acid extract was between 60% and 180% of the decrease in ash content caused by acid extraction.

Extraction of coals with hydrochloric acid significantly enhanced the amount of coals soluble in pH 8 buffer (Table 3). Initial amounts of coal solubilized were 145% to 675% greater for acid extracted coals than for water washed controls. In all cases, except for Wilcox, increased solubilities of acid extracted coals continued to be observed in all

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subsequent extractions. That is, it did not appear to be the case that each acid and water extracted coal had the same total alkali solubility and that the former was initially more readily solubilized.

Treatment of coals with.microbial cultures yielded mixed results. Both organisms used produced a yellow pigment with an absorbance maximum at 400nm and little absorbance at 500nm in controls that did not contain coal (data not shown). Because of this, biosolubility experiments were monitored by following absorbances at at 500nm. As control cultures aged, however, the pigment present began to change and absorb more at 500nm.

Mississippi Wilcox lignite was more readily biosolubilized than those other coals tested and did not behave as did other coals. As observed with alkali solubilization, acid treated Wilcox was more readily biosolubilized than water treated controls (Figure 1). It is not likely that the increased biosolubility of acid treated Wilcox was due to an increase in the amount of alkali produced because pH values of cultures containing water extracted Wilcox were consistantly one half pH unit greater than those observed for the acid extracted coal (data not shown).

Cultures containing Texas lignite, Wyodak or Beulah Zap became lightly colored after several days of incubation and then lost color as the experiment proceeded (data not shown). The initial colored material in solution appeared to be solubilized coal. It is unknown if the decolorization of these culture media in the latter stages of incubation represents soluble coal being metabolized and supression of pigment formation or if solubilized coal and extracellular pigments were being removed from solution (e.g., coprecipitated, absorbed...). Since the pH of all cultures were increased and maintained at values greater than 7.5 (data not shown), it is unlikley that coals became insoluble due to pH effects. The net result was that absorptions at 500nm increased initially and then decreased sharply (data not shown). Initial increases in absorbances were small (less than 0.1 absorption unit) but were consistantly greater in cultures containing acid treated Texas lignite, Wyodak or Beulah Zap than those observed in controls.

From these data, it appears that the removal of multivalent metal ions significantly enhances both the alkali- and biosolubility of coals without causing measurable oxidation. It appears that low-rank coals have two types of ash present: peripheral and intergral. Peripheral ash would be that ash present as inclusions (e.g., pyrite, clays, etc.) while integral ash would be that which is incorporated into the molecular structure of the coal as metal complexed with organic components of the coal (e.g., carboxylic acids, phenols, alcohols or their sulfur analogs). The presence of multivalent cations present in coal structure as metal complexes is consistent with the suggested structure for other low-rank coals (10). These metal complexes would be less soluble in dilute alkali and, because multivalent cations are involved, they may act as crosslinks between organic molecules in coal. If this working hypothesis is correct, then removal of integral ash by any method will result in lower molecular weights of coal organics and larger populations of free carboxylic acids, phenols and alcohols which will result in increased coal solubilities and greater opportunity for enzyme attack (bioconversion).

While alkali solubility has been shown to be a mechanism in coal biosolubilization, pyrolysis-mass spectral data has suggests that there may be another mechanism involved (11). This other mechanism may involve direct enzyme attack or it may involve the removal of metal ions by microbes. The illucidation of this other mechanism will be the subject of a subsequent manuscript.

ACKNOWLEDGEMENTS

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Table 1.   Analyses of Treated Coals.

Coal[a]	C[b]	H	N	S	O	Moisture	Ash
Wyodak-W	73.39	5.21	0.98	0.44	19.97	4.00	5.91
Wyodak-A	73.31	5.13	0.91	0.43	20.16	2.00	2.93
Δ				-0.01	+0.19		-2.98
TXL-W	71.61	4.89	0.92	0.68	21.90	5.31	7.95
TXL-A	70.76	4.76	0.97	0.73	22.78	4.41	2.69
Δ				+0.05	+0.88		-5.26
B.Z.-W	72.76	5.6	1.35	1.21	19.08	2.78	13.81
B.Z.-A	71.41	5.49	1.27	1.25	20.58	2.83	9.36
Δ				+0.04	+1.50		-4.45
Wilcoz-W	66.58	4.91	1.11	1.29	26.11	4.56	14.21
Wilcox-A	67.52	5.11	1.16	1.25	24.96	4.12	11.48
Δ				-0.04	-1.15		-2.73

a) Abbreviations are as follows: TXL - Texas lignite, B.Z. - Beulah Zap,
W - Water extracted, A - Acid extracted.
b) Values are the average of two determinations.
Δ are the difference between the control and experimental values.

Table 2.   Metal Concentrations Present in Coal Extracts.

Coal[a]	Al[b]	Ca	Fe	Mg	Na	K
Wyodak-W	0	0.5	0	0.1	3.6	0.3
Wyodak-A	31	243	36	35	17	1.5
TXL-W	0.1	15	0	5	1.6	0.5
TXL-A	36	453	139	66	9	4
B.Z.-W	0.1	0.3	0.1	0.1	12	0.2
B.Z.-A	29	255	34	69	89	3
Wilcox-W	3.6	41	9	5	0.5	0.5
Wilcox-A	95	427	725	23	5	4

a) Abbreviations are as in Table 1.
b) Concentrations are given as mg per liter.

Table 3. Solubilities of Coals in Tris Buffer, pH 8.0

Coal[a]	Treatment	First[b] Extraction	Second Extraction	Third Extraction
Wyodak	Water	0.126	0.164	0.101
Wyodak	Acid	0.532	0.388	0.243
TXL	Water	0.343	0.278	0.198
TXL	Acid	1.45	1.22	0.780
B.Z.	Water	3.78	0.326	0.152
B.Z.	Acid	25.5	1.58	0.800
Wilcox	Water	85.8	26.9	7.3
Wilcox	Acid	124	13.7	4.40

a) Abbreviations are as in Table 1.
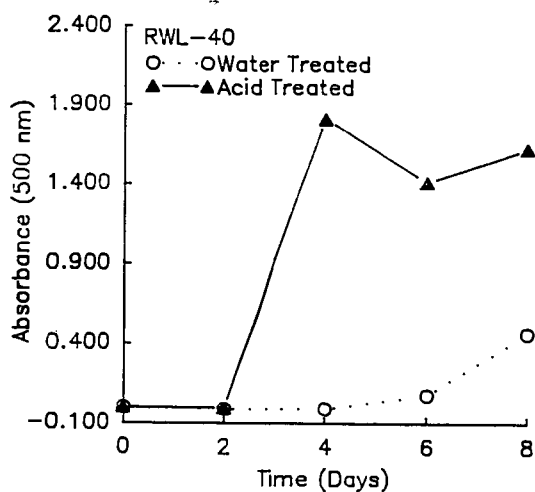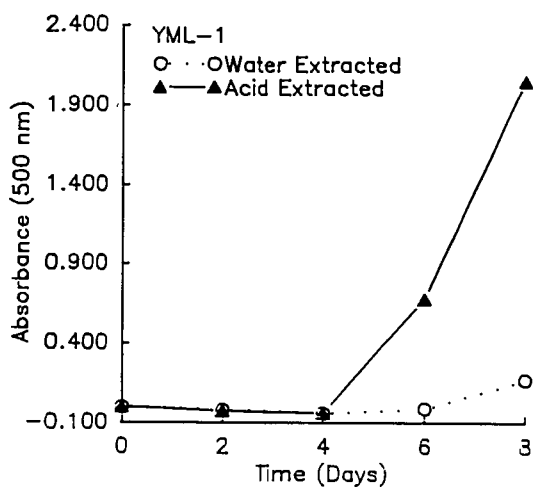b) Values are given as absorbance at 400nm (corrected for any dilution factors).

Figure 1. Biosolubilization of acid and water treated Mississippi Wilcox lignite.

MICROBIAL TREATMENT OF COALS AND ITS EFFECT
ON ASH FUSION PROPERTIES

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INTRODUCTION

Coal is a major source of energy in the United States, generating roughly 50% of the total electricity power produced, and 90% of the electricity used east of the Mississippi River. Coal usage for electrical power generation currently averages 550-600 million ton/yr, and is expected to increase despite diminishing quantities of high-quality (i.e., low sulfur content, low fouling) coals. Increasingly stringent regulatory standards for reduced levels of atmospheric sulfur emissions have increased demand for low-sulfur coal. The primary objection to high-sulfur coal is the consequent generation of sulfur oxides during combustion, which may play a major role in the formation of acid rain.

In addition to sulfur, the quantity of metals in the coal also affects coal quality. Metals which may be present, mostly in the form of minerals and sulfides, include Si, Fe, Ca, Mg, Al, Na, K, and Ti, as well as traces of As, Cd, Cr, Cu, Pb, Mn, Hg, Ni, and Zn. Coal combustion oxidizes these metals to produce significant quantities of coal ash, the disposal of which can be costly. Additionally, ash entrained with the hot combustion gas tends to foul heat exchange surfaces, which reduces furnace efficiency and causes operational difficulties. The presence of metals like Arsenic, Lead, and Mercury create problems in disposal of ash.

The most common techniques of coal benefication are physical separation methods which exploit density differences between the light organic coal and heavier inorganic impurities. Numerous chemical processes involving reaction and extraction have been proposed. However, these methods generally prove uneconomic due to the high temperature and pressure required to achieve desired removal of impurities. The use of microbial processes in various aspects of energy production has become increasingly important over the past several years. In particular, the isolation and characterization of bacteria which catalyze the solubilization of sulfur compounds in coal and petroleum has suggested the possibility of a biological approach to fossil fuel desulfurization.

Microbial desulfurization of coal presents some important advantages over physical/chemical processes. Ambient temperature and pressure are generally sufficient to promote good microbial growth. The microbes are self-regenerating, so the costs are minimal. Microbial processes offer the possibility of removing not only inorganic sulfur, but also organic sulfur in the coal.

Processes involving the microorganism Thiobacillus ferrooxidans have been considered as an alternative to desulfurization by physical and chemical methods. In addition to desulfurization, it may also be effective in removing pyritic iron, Ca, Mg, Al, Na, K, Pb, Mo, Zn, Cu, and Cr. This concurrent demetalization of the coal may enhance the relative economic attractiveness of microbial desulfurization by reducing the quantity of ash produced and improving the quality of the ash.

OBJECTIVES AND APPROACH

One objective of this study was to characterize the microbial demetalization and
desulfurization of coal by the bacterium <u>Thiobacillus ferrooxidans</u> under aerobic
conditions. The effects of type of coal, coal particle size, and nutrient con-
centration on the bacterial leaching of coal were investigated. Ash fusion
temperatures of treated and untreated coal samples were measured and an attempt
was made to relate variations to the coal and ash properties.

Another objective was to determine the extent of dissolution of different metals
from coal residues by the action of native autotrophic bacteria under aerobic
conditions. The effects of inorganic nutrient supplements were also studied.

RESULTS AND DISCUSSION

The major results from the aerobic demetalization and desulfurization tests on
three bituminous coal are summarized in Table 1. It is obvious that the effec-
tiveness of microbial treatment is dependent on the type of coal and coal ash.
More significantly, it can lead to substantial improvements in ash quality (as
defined by the ash fusion temperature) as seen with the Pittsburgh Coal.

Some resutls from aerobic dissolution of toxic metals from the fines fractions of
coal residues are summarized in Table 2. It is clear that under aerobic condi-
tions substantial fractions of other metals in addition to pyritic iron can be
solubilized from coal or coal residues.

CONCLUSIONS

Microbial action on bituminous coals under aerobic conditions can lead to sig-
nificant reductions in inorganic sulfur, metals and total ash content, as well as
considerable increase of ash fusion temperatures of the remaining ash.

Table 1

Coal	Ash Content (%)	Ash Reduction (%)	Inorganic Sulfur Reduction (%)	Increase in Ash Fusion Temperature (°F)
Ohio	25.2	15	80	180
Pittsburgh	10.3	17.5	40	460
Illinois	8.1	27	40	50

Table 2

Metal	Control	With No Nutrients Added	With Nutrients Added
		% Metal in Solution	
As	0.69	6.1	47
Cr	7.7	11	65
Cu	2.4	10	33
Fe (III)	0.1	2.7	26
Mn	16	16	43
Ni	15	20	44
Pb	0.6	0.6	4.6
Zn	8.2	17	48

SULFUR-SPECIFIC METABOLISM OF ORGANIC COMPOUNDS

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All fossil fuels (except most natural gas) contain sulfur. When these fuels are combusted, the sulfur present is released into the atmosphere contributing to air pollution in the form of "acid rain." Current air quality standards have already placed strict limitations on the amounts of sulfurous air emissions allowed, and these regulations may well become even more stringent in the future. There is then a need and an incentive to limit sulfurous emissions resulting from the combustion of fossil fuels. The current technology focuses on sulfur removal achieved during or after combustion. At this time there is no cost effective technology that can desulfurize fuel prior to combustion.

The cost of desulfurization equipment currently used to remove sulfur during and/or after combustion, the problems associated with maintaining that equipment, and disposing of the copious quantities of sulfurous wastes generated by these processes are expenses and problems that industry and the utilities would prefer to avoid. If clean low-sulfur fuel (other than natural gas) could be made available, then fuel users could avoid these concerns. Although there are naturally occurring sources of low-sulfur oil and coal, these high-quality fuels are not abundant enough to serve all of the world's energy needs. Biological processes occur under very mild reaction conditions compared to chemical reactions, so it is hoped that if a suitable means of desulfurizing fuels using biological systems can be found, then economically favorable pre-combustion fuel desulfurization processes will result.

It has been known for many years that acidic drainage associated with coal mines or coal storage piles is the result of microbial oxidation of sulfur in coal, which produces sulfuric acid. This microbial desulfurization of coal is almost completely limited to the inorganic forms of sulfur (pyrite) found in coal and is accomplished by the action of well, characterized bacteria: Thiobacillus ferrooxidans,   Thiobacillus thiooxidans,   and Sulfolobus acidocaldarius.   The microbiological removal of sulfur from coal has been shown to be capable of removing 90% or more of the inorganic sulfur, while the microbiological removal of organic sulfur from coal is certainly less complete and is less well-documented. More than 90% of the total sulfur must be removed for any pre-combustion coal desulfurization process to obviate the need for subsequent desulfurization. Therefore, the focus of current research concerning the microbiological removal of sulfur from coal centers on the removal of organic sulfur.

Inorganic sulfur generally exists in coal in the form of discrete particles or crystals of pyrite; whereas organic sulfur occurs as an integral part of the molecular coal matrix and is not readily accessible for microbial attack. The goal, then, of developing a microbiological process for the removal of organic sulfur from coal — while retaining the fuel value of that coal — is a most difficult goal indeed. What is needed are microorganisms that have an affinity for cleaving carbon-sulfur bonds, that is, microorganisms capable of sulfur-specific metabolism of organic compounds.

Enrichment culture techniques are typically used to isolate microorgan-
isms with desired traits, and the search for microorganisms capable of organic
sulfur removal from coal is no exception. The chemical dibenzothiophene (DBT)
is generally regarded as a good model compound representative of organic
sulfur found in coal; it is used as the substrate of choice in most enrichment
culture experiments. There are two fundamentally different pathways for the
microbial degradation of DBT that have been proposed. Both pathways are
outlined in Figure 1. Pathway A (the sulfoxide/sulfone/sulfonate/sulfate "4S"
pathway) is the sulfur-specific metabolic pathway that results in the removal
of sulfur from DBT, while leaving the carbon intact. Pathway B is a carbon-
destructive metabolic pathway which results in the overall degradation of
DBT. Numerous investigators[1-6] have attempted to isolate or develop microbial
cultures that can metabolize or co-metabolize DBT. A summary of the published
results of these attempts to isolate DBT-utilizing cultures is presented in
Table 1. The nearly universal experience has been the isolation of micro-
organisms that metabolize DBT by the carbon-destructive pathway. The
implication is that the methods of screening bacterial cultures and of
performing enrichment culture experiments may be inappropriate and in need of
revision. The published reports concerning CB1,[6] the only bacterial strain
claimed to metabolize DBT via Pathway A exclusively, do not give any
indication of a novel culture enrichment procedure or offer an example of how
to purposefully isolate microorganisms with sulfur-specific metabolic
capabilities.

There are several major research needs that can clearly be identified at
this time. Since the rate and extent of organic sulfur removal from coal by
characterized microorganisms is insufficient to warrant process development at
this time, there is a need to identify new microorganisms that have sulfur-
specific degradation abilities toward the full array of sulfur-containing
organic molecules. Toward the isolation of new bacterial strains with
appropriate desulfurization activities, there is a need for a convenient and
powerful strain selection technique rather than relying on laborious culture
screening techniques. There also exists a need to compare the cultures and
the results obtained from different laboratories concerning microbial
desulfurization. We need a way to normalize the results obtained by different
researchers, using different microorganisms, different substrates, and
different growth/reaction conditions. Existing strains of bacteria with
documented desulfurization abilities are in need of strain improvement and
techniques to conveniently monitor the progress of strain improvement efforts.
Lastly, there is a need for better analytical techniques to assess the removal
of organic sulfur from coal, as the existing methods of physical/chemical
analysis of sulfur by type in coal are costly, time consuming, and not
particularly accurate — especially regarding the organic sulfur in coal. All
of these research needs can be served by taking advantage of the fact that all
living organisms require sulfur for growth.

Enrichment cultures can be established to isolate microorganisms that
possess an array of characteristics reflective of the incredible diversity
present in the microbial kingdom. The challenge is to manipulate the culture
conditions such that a very powerful selective pressure exists favoring only
those microorganisms that possess the desired trait; then all that remains is
to provide a suitable inoculum and allow the selective pressure and the
"survival of the fittest" to interact and allow only those microbes that
possess the desired characteristic to predominate in the enrichment culture.
Enrichment cultures are startlingly simple in theory. The compound to be
degraded is supplied as the growth-limiting source of an essential nutrient,

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while all other growth requirements are supplied in abundance. Growth under
such conditions favors those microorganisms that possess degradative
activities toward the target compound — whereby the "appropriate" micro-
organisms should outgrow all "inappropriate" competitors and rapidly come to
dominate the culture. A growth limitation can be achieved by removing,
reducing, substituting, or altering any essential nutrient; however, in
practice it is usually only the carbon source that is manipulated in enrich-
ment culture experiments. That also seems to be the case for enrichment
culture experiments reported for the isolation of microorganisms with the
ability to desulfurize DBT or the organic sulfur in coal. It would be much
more to the point to establish enrichment cultures utilizing DBT as the
growth-limiting source of sulfur.

Enrichment culture experiments were carried out at the Institute of Gas
Technology (IGT) in two ways: traditional shake flask enrichment cultures and
sulfur-limited chemostats. A sulfur-limited chemostat is a continuous culture
bacterial growth experiment in which all of the nutrients, except sulfur, that
are needed for growth are supplied in abundance, and sulfur is supplied in the
form of coal or organic chemicals. The use of traditional shake flask enrich-
ment culture techniques resulted in the isolation of a Pseudomonas species,
designated TG-232, which is capable of co-metabolizing DBT while growing at
the expense of naphthalene. The sulfur-limited coal chemostat resulted in the
isolation of a mixed bacterial culture, designated IGT-S7, capable of co-
metabolizing DBT while growing at the expense of glucose, succinate, or
benzoic acid. The bacterial metabolites of DBT produced by TG-232 and IGT-S7
were identified by gas chromatography and mass spectrometry (GC/MS). The
results from these analyses are presented in Table 2. It can readily be seen
that TG-232 metabolizes DBT by the carbon-destructive Pathway B, while IGT-S7
is capable of sulfur-specific metabolism, which removes sulfur from DBT and
leaves the carbon intact. Sulfur-limited chemostats appear to be a useful
tool for the purposeful isolation of microorganisms with sulfur-specific
metabolic capabilities.

Growth of cultures in a sulfur-limited medium is not only useful for the
selection of microorganisms but also the sulfur requirement of all micro-
organisms for growth can be used as the basis of a quantitative sulfur-
specific bioassay by correlating bacterial growth to substrate metabolism. A
bioassay can detect sulfur utilization from organic substrates more easily and
with greater sensitivity than physical/chemical analyses. Moreover, a
bioassay can be used to compare the results of different bacterial strains
acting on different substrates. A bioassay relating sulfur metabolism of
organic substrates to the rate and extent of growth observed can be a
universally applicable method useful in normalizing the data obtained from
every researcher in the field of microbial desulfurization of coal.

Because all life requires some amount of sulfur for growth, a situation
can be created such that by quantifying bacterial growth one can quantify the
utilization of any organic or inorganic compound as a source of sulfur. In
work funded by the U.S. Department of Energy, IGT developed a sulfur bioassay
to do just that. An inorganic sulfate is usually the form in which sulfur is
supplied in a bacterial growth medium. Similarly, various organic sulfur
compounds can be tested for their ability to serve as sources of sulfur for
bacterial growth. Growth curves of IGT-S7 with glucose serving as a carbon
source and various sulfur sources (nothing, sulfate, DBT, and trithiane) are
presented in Figure 2. These curves, which constitute sulfur bioavailability
assays, illustrate several points. The no-sulfate control shows some growth

because trace amounts of sulfur are present in this medium, but this growth is comparatively slow and essentially complete after 24 hours. The experiment that contains sulfate shows extremely rapid growth that is largely complete within 8 hours. When the same bacterial culture is grown with either DBT or trithiane as sulfur sources, about 78 hours are required to attain maximal growth. Inspection of these growth curves reveals that the bacterial cultures that contain DBT or trithiane grow at approximately the same rate as the no-sulfate control for the first 24 hours. This suggests that DBT and trithiane are not utilized appreciably as sulfur sources until the trace amounts of sulfur compounds present in the medium have been exhausted. Thereafter, a slow rate of growth continues at the expense of the organic sulfur compounds. Moreover, there is no pronounced lag in the growth curves of the DBT or the trithiane cultures after 24 hours coinciding with the exhaustion of the trace sulfur in the medium. This implies that no lengthy induction/adaptation period is required for IGT-S7 to utilize organic sulfur sources.

IGT-S7 is capable of utilizing a variety of organic compounds as sulfur sources including, but not limited to, dibenzothiophene, dibenzothiophene sulfoxide, dibenzothiophene sulfone, thianthrene, thioxanthene, and trithiane. While all of these compounds can be used as sulfur sources by IGT-S7, the time required to achieve maximal cell growth is longer than when sulfate is present. However, when bacterial cultures capable of metabolizing DBT by Pathway B are tested in the sulfur bioavailability assay, the vast majority of these cultures show no growth whatsoever in excess of that observed in the "no-sulfur controls." TG-232 is capable of utilizing DBT as a sulfur source, but it never grows to more than 40% of the maximum growth observed in the sulfate-containing controls, even upon lengthy incubation and multiple sequential subculturing. The sulfur bioavailability assay, therefore, is a convenient inexpensive method to compare quantitatively the abilities of bacterial cultures to utilize organic substrates as sulfur sources.

When IGT-S7 is used to treat coal, as much as 25% removal of organic sulfur has been observed. These results are encouraging, but organic sulfur removal efficiencies of about 90% are required if a microbial coal desulfur-ization process is to be developed that is competitive with current post-combustion desulfurization technologies. Significant strain improvement research is needed to enhance the desulfurization activity in bacterial cultures, and physical problems associated with the pre-combustion desulfuri-zation of coal must be addressed if a viable technology for the microbial removal of organic sulfur from coal is to be realized.

Sulfur-limited continuous culture chemostats can be used to isolate/develop bacterial cultures capable of sulfur-specific metabolism of organic compounds. Similarly, the sulfur bioavailability assay can be used to evaluate quantitatively the desulfurization abilities of bacterial cultures and can aid in culture evaluation and strain improvement experiments. If the goal of developing a microbiological process for the removal of sulfur from coal is to be achieved, bacterial cultures with expanded and improved abilities to desulfurize organic substrates are needed. The techniques described in this paper are useful tools that can help to achieve that goal.

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Table 1. SUMMARY OF THE IDENTIFICATION OF DBT METABOLITES
PRODUCED BY MICROBIAL DEGRADATION

Research Group	Pathway A Degradation		Pathway B Degradation	Most Complete Degradation Products Observed[*]
	————————	%	—————————	
Kodama et al.[5]	8		92	A1, B3
Laborde & Gibson[3]	Minor		Major	A1, B3
Monticello et al.[4]	0		100	B3
Kilbane: TG-232	32		68	A1, B6, B10
IGT-S7	100		0	A4
Hou & Laskin[2]	0		100	B2
Malik & Claus[1]	0		100	B2
Isbister & Kobylinski[6]	100		0	A4

[*] The designations of A1, B1, etc. refer to structures included in the
pathways presented in Figure 1.

Table 2. GC/MS ANALYSIS OF BACTERIAL-DERIVED METABOLITES OF DBT

The most abundant metabolites of DBT degradation were analyzed by GC/MS. The
concentration of DBT was arbitrarily set at 1.0, and the concentrations of
metabolites were reported relative to the concentration of DBT. The numbers
in parenthesis (A1, B1, etc.) refer to structures included in the pathways
presented in Figure 1.

Compound	Mol. wt	TG-232	IGT-S7
Dibenzothiophene	184	1	1
(A1) Dibenzothiophene-5-oxide	200	0.30	1.8
plus Phenoxathiin	200	--	--
(A4)[*] Dihydroxybiphenyl	186	BDL[**]	0.033
(A4)[*] Hydroxybiphenyl	170	BDL	59
(B3) 3-hydroxy-2-formyl-benzothiophene	178	0.21	BDL
Biphenyl	154	BDL	0.001
(B6) Benzothiophene	134	0.016	BDL
Naphthalene	128	0.001	BDL
(B4) Three isomers of $C(8)H(6)OS$:			
(hydroxybenzothiophene)			
No. 1	150	0.01	BDL
No. 2	150	0.02	BDL
No. 3	150	0.048	BDL
(B9) $C(9)H(8)OS$	164	0.12	BDL
(B7) $C(9)H(8)O(2)S$	180	0.067	BDL
(B5) $C(9)H(6)OS$	162	0.022	BDL
$C(10)H(10)OS$ or $C(9)H(6)O(2)S$	178	0.035	BDL
(B10) $C(8)H(8)O(2)S$ isomers a)	168	0.033	BDL
b)	168	0.025	BDL
Formula (?)	220	0.036	BDL
Total Excluding DBT		0.94	60.8

[*] Hydroxybiphenyl is the terminal metabolite in a modified A/"4S" pathway.
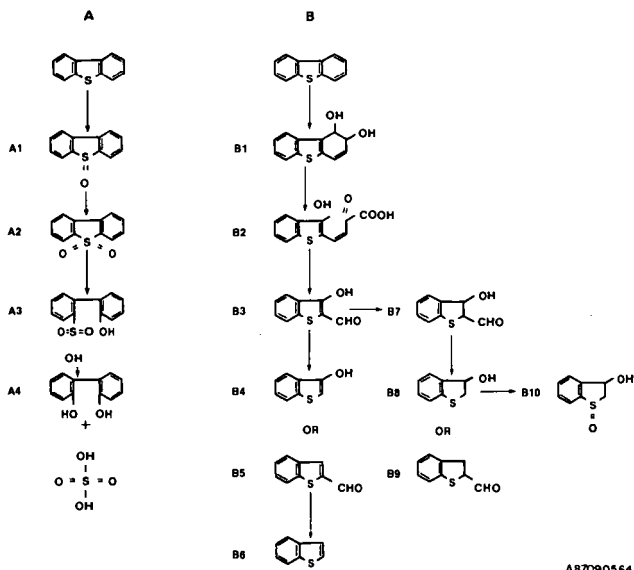[**] BDL = below detection limit.
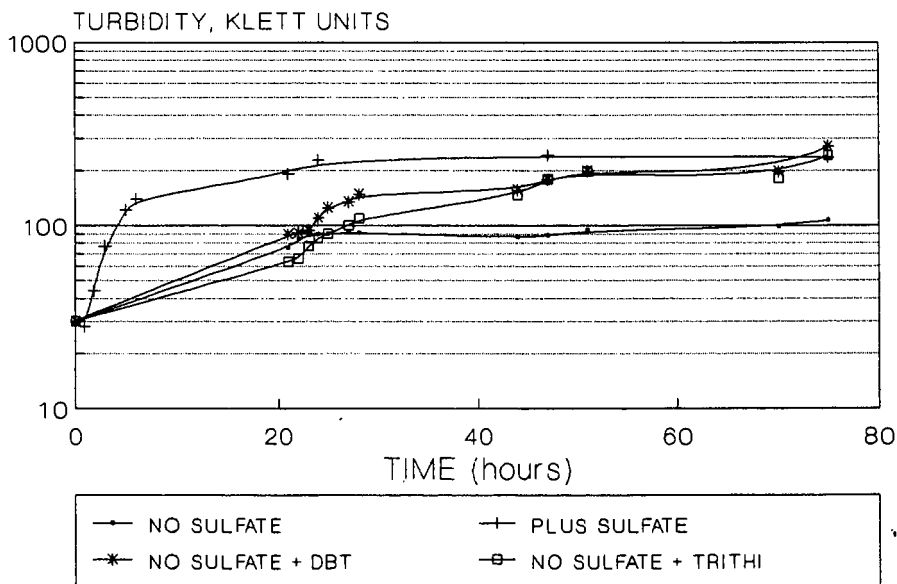
Figure 1. PROPOSED PATHWAY OF DBT DEGRADATION

Figure 2. GROWTH CURVES OF IGT-S7 USING ORGANIC AND INORGANIC SULFUR SOURCES

SOLUBILIZATION OF AUSTRALIAN LIGNITES BY MICROORGANISMS

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INTRODUCTION

Cohen and Gabriel (1982) found that the wood rot fungi
Polyporus versicolor (Trametes versicolor) and *Poria
monticola (Poria placenta)* were able to solubilize
Leonardite, a naturally oxidized lignite from a deposit in
North Dakota. Lignite pieces placed on the surface of
mycelial mats of each species, growing on Sabourauq maltose
agar, were reduced to black water soluble products, shown by
Wilson *et al* (1987) to contain aromatic, carboxylic and
aliphatic carbon and have molecular weights of 10,000 to
50,000 Daltons or more.

Other North American lignites were found to be much less
susceptible to solubilization by both these and other fungi
(Ward 1985_1 and Scott *et al* 1986). However, Strandberg and
Lewis (1986) showed that a range of lignites and a
subbituminous coal could be solubilized by Candida (ML13)
following oxidation with acid, peroxide or ozone. Several
lignin degrading organisms have also been shown to
solubilize oxidized lignite, including: *Phanerochaete
chrysosporium* (Scott and Lewis 1987), *Streptomyces setonii*
and *S. viridosporus* (Strandberg and Lewis 1987). Ward
(1985_2) was able to isolate a range of lignite degrading
fungi from a naturally exposed lignite seam. These included
species of Aspergillus, Candida, Mucor, Paecillomyces and
Penicillium. The mechanisms involved are not clear and may
not all reflect enzymatic breakdown of lignin like
structures remaining in lignite. Fakoussa and Truper (1983)
have reported that surfactants produced by the bacterium
Pseudomonao fluorescens have a solubilizing action on coal.

Australia has substantial lignite deposits, particularly in
the Latrobe Valley in Victoria where 4.10^{10} tonnes are
accessible with available technologies (Perry *et al* 1984).
We have investigated the susceptibility of these coals to
solubilization by microorganisms, including species
additional to those already identified as active on North
American lignites.

MATERIALS AND METHODS

Cultures were obtained from: The American Type Culture
Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC
11538, 12679 and 24725); CSIRO Division of Chemical and Wood

Technology, P.O. Box 56, Highott, Victoria 3190, Australia (DFP 7522); Dr. G.W. Strandberg, Chemical Technology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA. *(Streptomyces viridosporus)*; and Dr. B. Ward, Department of Biology, University of Mississippi, University, MS 38667, USA (ML-13, ML-20 and YML-1).

Solubilization tests were conducted at 28°, at a relative humidity exceeding 80%, in 250ml screw capped jars containing 50ml of Sabouraud maltose agar. Tho jar closures had a 10mm perforation plugged with non-absorbent cotton. For *Trametes versicolor*, lignite pieces were placed on the surface of the mycelial mat, 8 days after central inoculation of the medium. For other tests, coal pieces were added at the same time as a spread inoculum of a suspension of conidia or mycelial fragments. Lignite solubilization was detected by the appearance of pigmented droplets on the surface of the coal pieces and by diffusion of dark solubilization products into the medium. The degree of solubilization was quantitated by recovery of remaining lignite pieces which were washed in deionized water, dried at 95° and weighed.

Lignite samples were obtained from the Coal Corporation of Victoria. Run of mine coal came from a 20,0001 sample taken at Morwell in October 1982 and a 40,0001 sample taken at Loy Yang in October 1984. Both had been stored in sealed drums. Weathered lignite came from a deposit at Loy Yang. The samples were pushed through a 4mm screen and fines passing a 2.4mm mesh removed. Lignite was sterilized by autoclaving at 121° and dried to constant weight at 95° prior to use. Lignite was oxidized by reacting with 8M nitric acid for 18 hours, washed with water and dried. Approximately 300mg dry weight of lignite was used for each test.

RESULTS

Mycelial mats of *Trametes versicolor* were able to solubilize both Latrobe Valley lignites following acid oxidation (Table-1). Run of mine lignite was resistant, with the exception that Loy Yang coal showed trace coloration of droplets forming on the coal pieces and diffusion of dark material into the agar surrounding coal fragments. Pigmented droplets formed within 14 days both on acid oxidized Morwell and acid oxidized Loy Yang lignites. There was extensive collapse of the granules by day 38. The incubation was continued to day 118 prior to quantitation. Although pigmented droplets appeared on the Loy Yang run of mine coal at 42 days, there was no extensive solubilization. Mass gain by unoxidized lignite pieces reflects the difficulty of separating them from the pigmented droplets formed and no pigments diffused into the agar in cultures without lignite or when lignite was in contact with medium under aseptic conditions.

Seven strains of six additional species were tested for their effect on acid oxidized and run of mine Morwell lignite, and on a naturally oxidized, weathered, lignite deposit at Loy Yang, Table-2. Each of these species, previously shown to be active on oxidized North American lignites, were able to solubilize acid oxidized Morwell lignite. *Poria placenta* was least effective and the ATCC 11538 strain reported active on Leonardite (Cohen and Gabriel 1982) did not solubilize any of the Latrobe Valley lignites in repeated tests. A strain of *P. placenta* from the CSIRO collection showed some activity on acid oxidized Morwell coal by the diffusion test. Each of the other five species caused extensive or total solubilization of acid oxidized lignite and were also active on the naturally oxidized weathered coal on which *Phanerochaete chrysosporium* was the most effective. None of the isolates tested showed evidence of activity on run of mine coal, with the exception of Candida ML-13 that was positive by the diffusion test but so heavily interpenetrated the coal grains that quantitation of the extent of solubilization was not possible.

DISCUSSION

The data presented here show that acid oxidized lignites from the Latrobe Valley are solubilized by each of seven species of microorganisms previously found to be active on Leonardite and oxidized North American lignites. These are the wood rot fungi: *Trametes versicolor, Poria placenta and Phanerochaete chrysosporium,* the lignin degrading prokaryote *Streptomyces viridosporus* and three fungi isolated from lignite in Mississippi: Candida ML-13, Cunninghamelia YML-1 and *Penicillium waksmanii*.

The completeness of solubilization by these organisms was not correlated with the time of onset of solubilization as indicated by the diffusion of pigment from lignite grains, data not shown, and varied from as soon as 5 days after inoculation in Cunninghamella, that gave 74% solubilization by day 67, to as late as 46 days after inoculation by *Penicillium waksmanii* which gave 100% solubilization by day 67. This suggests that there is substantial potential for the manipulation of conditions and of the organisms themselves to speed solubilization. It should be noted that the extended incubation periods used in these tests do not reflect the speed of the process by the most active species and were chosen such that weak or delayed activity could be detected. It is desirable that even slow acting isolates are identified since they may include species degrading lignite by different mechanisms. Identification of all biological mechanisms of lignite degradation may provide pathways to desirable products that can be manipulated to give good yields at practicable rates.

Further to the work reported here, we have surveyed other
lignin and wood degrading species for their activity on
Latrobe Valley lignites. Of the lignin degraders, four were
inactive but the remaining six solubilized acid oxidized
Morwell lignite and also weathered lignite to a substantial
extent. Three of these species also showed some activity on
run of mine Morwell lignite. In addition, we have
established enrichment cultures for lignite degrading
species from natural lignite exposures and from mining sites
in Victoria. These enrichments have yielded a wide range
fungi, Streptomycetes and bacteria able to grow on lignite
as their sole carbon source. The isolates include some able
to quantitatively solubilize acid oxidized Morwell lignite
and some that partially solubilize unoxidized, run of mine
Morwell coal. This work has identified a second deposit of
lignite at Loy Yang, that like Leonardite is readily
solubilized by microorganisms without further treatment.

ACKNOWLEDGEMENTS

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TABLE-1   ACTION OF *TRAMETES VERSICOLOR* ON RUN OF MINE AND ACID
           OXIDIZED AUSTRALIAN LIGNITES.

LIGNITE		COLOURATION OF		MASS CHANGE OF COAL %
		DROPLETS ON COAL	AGAR	
MORWELL	ROM	NONE	NONE	+5
	OXIDIZED	BLACK	BLACK	-90
LOY YANG	ROM	TRACE	TRACE	+4
	OXIDIZED	BLACK	BLACK	-83

TABLE-2   SOLUBILIZATION OF MORWELL AND LOY YANG LIGNITE BY
           MICROORGANISMS.

		LIGNITE					
		MORWELL ROM		MORWELL OXIDIZED		LOY YANG WEATHERED	
		a	*b*	*a*	*b*	*a*	*b*
Candida sp.	ML-13	B	+14	B	-74	B	-13
Cunninghamella sp.	YML-1	0	+1	B	-74	B	-1
Phanerochaete chrysosporium	ATCC24725	0	+1	B	-35	B	-47
Poria placenta	ATCC11538	0		0		0	
	DFP7522	0		B		0	
Penicillium waksmanii	ML-20	0	+2	B	-100	B	-3
Streptomyces viridosporus	T7A	0	+9	B	-75	B	-10

a   Formation of pigmented droplets on coal pieces and/or
       diffusion of black pigment from the coal into the agar:
       0 not observed,   B black pigment.

b   Percentage mass change of lignite pieces following 58
       to 70 days incubation. Apparent mass gain is due to
       mycelium penetrating the lignite pieces.

CONSIDERATIONS FOR THE
COMMERCIALIZATION OF COAL BIODESULFURIZATION
TECHNOLOGY

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INTRODUCTION

The adverse effects to human health and to the ecological
environment caused by the release of sulfur dioxide (SO2) to
the atmosphere during coal combustion have gained increasing
attention due to the acid rain debate. Control of SO2
emissions, however, have been regulated under various federal,
state, and local standards for well over ten years. The 1977
amendments to the Clean Air Act (CAA) introduced Environmental
Protection Agency (EPA) regulation of new utility coal-fired
boiler emissions under the New Source Performance Standards
(NSPS) of 0.6 lbs/mm BTU. Compliance with these standards and
National Ambient Air Quality Standards (NAAQS) have required
that many utilities purchase coal based on sulfur content,
often more expensive feedstocks, and also invest in costly flue
gas desulfurization processes, especially scrubbers.

Currently the EPA is in the midst of promulgating SO2 control
regulations for the industrial boiler market, which mandate a
90 percent reduction of emissions from all new coal industrial
and commercial boilers regardless of the sulfur content of the
coal. Compliance with these regulations may prove very
difficult for industrial boiler owners. For example, boiler
emissions would have to be lowered to 0.6 lbs/mm BTU when using
3 percent sulfur eastern coal. This would cause fuel switching
to natural gas, rather than expensive coal cleaning or flue gas
treatments.

The pre- or post-combustion removal of sulfur from coal,
therefore, is a regulatory driven necessity but is constrained
by technological and economic difficulties. Technologies that
are commercially available include a variety of physical and
chemical coal cleaning techniques and several flue gas
treatment processes. Table 1 lists the most common,
commercially available technologies with an approximation of
their efficiencies and costs. As shown, both capital and
operating costs for these processes are quite significant, and
in most cases remove less sulfur than required to meet
regulations. The successful commercial introduction of any new
technology into this marketplace, therefore, requires both
efficient sulfur removal and cost competitiveness.

BIODESULFURIZATION OF COAL

One technology for coal desulfurization that is under
development, but still far from a commercial reality, is
biological treatment. In the past few years, several studies
have identified the technical status of coal biodesulfurization
research, listing published results of laboratory scale
experiments both in the United States and overseas (National
Bureau of Standards, 1986; Battelle Pacific Northwest
Laboratories, 1986; Couch, 1987). These studies refer to
experimental results published since 1914, although the
majority have appeared in the past twelve years. Most of these
experiments have been concerned with the microbiology and coal
chemistry aspects of coal biodesulfurization and very few have
looked at the engineering, process, and economics aspects.
Notable examples of this latter category include studies by Bos
(1985), Dugan (1985), and Sproull (1986).

Results from these studies indicate that pyritic sulfur removal
using the acidophilic organism Thiobacillus Ferroxidans has
been achieved widely, with over 90 percent removal reported by
many researchers. In addition, these sulfur oxidizing microbes
remove ash and some metals during the biodesulfurization
process. Removal of organic sulfur is more difficult; the use
of thermophilic organisms have apparently reduced the organic
sulfur component of some high sulfur coals by up to 30 percent
(Chandra, 1987; Isbister 1986). What is clear from these
studies is that biological treatment provides no utopian
solution to the coal desulfurization problem and may only be
part of a more complex, multifaceted process. Furthermore,
biological treatments may be more suitable for some coals than
others and may not deal with both the organic and inorganic
sulfur present in coal. On the other hand, there is some
indication that biological removal of pyrite may be
economically competitive with physical coal cleaning methods,
especially as operations and maintenance costs will be limited
and a greater percentage of sulfur will be removed.

Given this current status, therefore, what can be done to bring
coal biodesulfurization technology to the marketplace within a
timeframe that is compatible with the regulatory drivers? In
answering this question, it is important to understand the
prominent issues impeding the progress of coal bioprocessing
research:

*   Transfer of fundamental studies to pilot or
    demonstration activity -- emphasis remains on
    benchtop studies and, as shown in Figure 1, only
    about 15 percent of these have given any attention to
    either process design, engineering, or economics.
*   Low level of research funding and emphasis --although
    clean coal technologies are receiving large-scale

funding from the government, the Electric Power
Research Institute (EPRI), and the private sector,
fundamental research in biotechnology lags far behind
the funding levels available for demonstration
activities in other coal cleaning areas, flue gas
treatment processes, fluidized bed combustion, or
fuel cells.
- Lack of commercial biotechnology company interest --
unlike the pharmaceutical, agricultural, or metals
extraction industries, coal bioprocessing leads to a
low value-added product.

Overcoming the first of these impediments by transferring
fundamental studies to demonstration activity will probably
remove the remaining two. Quite often, not all of the
components of a given process are fully developed by the
inventors, but the overall concept has merit and should be
tested at a larger scale. Some see this approach as risky,
which it is, while others see it as an opportunity to begin
tackling the next phase of problems. Hopefully, by the time
the process is brought to commercialization, an acceptable
solution to all of the "underdeveloped" portions of the
technology have caught up. In making a case for early scale
up, this paper presents examples of several industries and
their own progress in commercializing biological processing.
By offering these examples and comparing them to coal, it is
the objective of this paper to stress performance of both
applied and fundamental research early on in order to
accelerate the implementation of a commercially viable process.

BENCH TO COMMERCIALIZATION -- INDUSTRY EXAMPLES

The importance of achieving a synergism between applied
technology and fundamental microbiology was poignantly
exemplified in the petroleum industry. When the development of
the petroleum industry was in its infancy, neither the
petroleum, technologists nor the microbiologists were aware of
the others activities (Davis, 1967). It was in fact a Russian
geologist who is credited for using bacterial hydrocarbon
oxidation as a prospecting tool. He had enlisted the
assistance of microbiologists who were working separately on
the same problem, and their joint efforts resulted in a
practical solution for petroleum exploration. In a similar
fashion during the 1940's, petroleum geologists in the United
States established the need to involve microbiologists to
resolve issues relating to source sediments. In this case as
well, those involved with the practical application solicited
the assistance of those with a technical understanding of
microbial interactions and, together, they engineered a timely
and workable solution.

Another example involves waste water management. Even though
the biological management of wastes, particularly waste waters,
has been employed for centuries, a firm understanding of the

principles has only been achieved in recent decades. Engineers, aided by available biological data, have successfully designed and implemented scores of aerobic waste water treatment systems, and new systems are still in the making. The proper engineering and optimization of biological water treatment systems are continuing to improve the industry as evidenced by the anaerobic bio-reactors now being developed for municipal waste water treatment (Cheremisinoff, 1987).

In the agricultural area, processes for improving crop yields and reducing infestation have also been approached with the fundamental microbiology and genetic engineering research occurring at the same time as the applied system development and the overcoming of policy and regulatory constraints.

The metals mining industry provides one of the most striking examples in recent years of a large, industrial-scale microbiological process that has been snatched from the laboratory beaker and engineered into production before "the ideal" set of microbiological data has been documented.

Much of the practical beginnings of bioextractive metallurgy in the copper industry is attributed to J. Prater, E. Malouf and other engineers at Kennecott Copper Corporation (now BP Minerals America) who, in the 1960's, initiated a dump leaching operation at Bingham Canyon to recover metal values contained in sulfide copper ores. Gleaning what biological information they needed, they successfully engineered and implemented one of the world's first and largest dump leaching operations. Initially, these engineers knew relatively little about how optimal strains of bacteria performed on model compounds, and what conditions might produce the optimal response during leaching. Nevertheless, from their efforts to get the wheel rolling, as well as those of others like them, bioleaching operations in copper and uranium have spread throughout the world. It was the iterative process of engineering design, fed by increased microbiological understanding, that led to the successful implementation of the technology.

In a similar vein, precious metals recovery from sulfide minerals made great strides once the process moved from laboratory investigation to pilot-plant development studies. A number of investigators had been involved in fundamental studies of the biooxidation of pyrite so that encapsulated gold particles could be liberated for subsequent cyanidation (Bruynesteyn, 1984; Lawrence, et. al., 1985; and Murr, et. al., 1980). A few engineering, research, and equipment companies over the past 10 years have undertaken pilot-plant scale-ups of biooxidation systems for precious metal recovery by drawing on the available biological data. Coming to grips with engineering issues such as reactor design, slurry dewatering, effluent water treatment, reagent and nutrient consumption, aeration requirements, materials of construction and interfacing the biooxidation process with pre- and post-

treatment processes have led to the installation and operation of at least three commercial plants in the free world.

Feasibility studies also played an important role in establishing the economic viability and eventual plant implementation of bacterially-enhanced precious metals recovery (Marchant, 1986). It was the present author's experience that the potential for a significant return on investment by biologically treating gold-bearing concentrates prompted a large scale pilot-plant study (Gilbert, et. al., 1988). Certainly, microbiological studies are evolving along with the economics and the practical plant applications, but with a better focus toward the real needs of the process.

LESSONS FOR COAL BIODESULFURIZATION

From the above examples it can be seen that the speed of progress in bringing biotechnology to a commercial reality resulted from early scale up or applied research supporting fundamental studies. Among the reasons for this appear to be motivation and approach, especially evident in the metals extraction area. In that case, the value added by the process was relatively high, especially in bioleaching of gold and other precious metals. Further, due to this motivation of producing a valuable product, the approach to developing an efficient technology focused foremost on the process engineering and economics, utilizing available microorganisms in increasingly larger-scale demonstration programs. As a feasible process became evident, refinements to the process based on an improved understanding of the microbiology and chemistry were made.

Biodesulfurization of coal is now at the stage where pilot plant efforts should begin. By placing the carrot, cart and horse in proper order, productive progress can be made. At present, there is a shortage of engineering data to keep pace with the fundamental microbiological studies now in progress. The bulk of governmental and industrial research support has been focused on microbiological developments dealing with isolated bacteria, model compounds, and sterile coal samples. Progress and funding are now slowing so that in a few years the process development of biodesulfurization might be strictly an academic issue. A solid pilot-scale study accompanied by a thorough economical feasibility study is needed to revitalize the program.

Scale up of coal biodesulfurization experiments will provide essential data about the feasibility of such a process and its application to the numerous coal feedstocks in the market. By applying what is already known, researchers can answer some of the fundamental engineering and economics concerns that will either make or break further efforts in this field. For example:

- Can bioextraction handle large tonnages of coal?
  Even for industrial boiler application, the volumes
  of feedstocks involved are far greater than in other
  biotechnology applications.
- If a superbug is developed that can reduce greater
  levels of organic sulfur and/or pyritic sulfur will
  it thrive in the engineered environment?
- What are the pre- and post-treatment requirements?
- What are the environmental benefits and concerns
  relating to solids, liquids, and gasses involved with
  the process? What are the volumes of each?
- Is it economical to treat run-of-mine coal or
  possibly a reject stream?
- Can organic and inorganic be coupled together in the
  same stage, or handled separately?
- What are the supply and cost constraints of
  nutrients?
- What impact will coal biodesulfurization have on the
  overall power plant design and performance?

In terms of pyrite removal, a technical feasibility study of
coal biodesulfurization carried out in the Netherlands (Bos,
1985) suggests that the process is a realistic, economic
option, although probably in conjunction with other processes.
Bos concluded the following:

- Coal must be milled extensively to achieve an
  acceptable pyrite removal without considerable carbon
  loss.
- Pyrite removal was achieved on all of the 17
  different, worldwide coal samples tested. In
  addition heavy metals and varying amounts of ash were
  removed.
- A temperature of 30 degree C. is optiomal for T.
  Ferrooxidans cultures, but residence times for 90
  percent pyrite removal are between 7 to 9 days.
- Long residence times and large tonnages of coal will
  require large reactors. He recommends a trough-
  shaped system similar in cross section to the
  Pachuca-tank reactor.
- For the treatment of 1 ton of coal in a 1 million
  tons of coal per year installation costs range
  between $9 - 16.

These results and others presented by Dugan and Sproull
certainly suggest further feasibility analyses and scale up
studies. In particular, economics data that include the costs
and benefits of combined sulfur, ash, and metals removal must
be carefully evaluated.

In conclusion, microbial studies have brought us a long way,
but they will not satisfy the need to demonstrate the
technology at a larger scale. Answering the engineering
questions could well be the bucket of cold water that douses

degree has been earned but experience is needed. It is up to the research community to gain the experience from scale up and demonstration programs before earning a higher degree in the fields of microbiology and chemistry.

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Table 1: Commercially Available Coal Desulfurization Technologies

TECHNOLOGY	PERCENTAGE SULFUR REMOVAL	OP. COST ($/T)	CAP. COST ($/KWE)
TRW Gravimelt	80-90	30	82
Flue Gas Desulfurization	Up to 90	7-12	150-200
Physical Coal Cleaning	30-40	4-6	50
Advanced Flotation	53	25	110
Oil Agglomeration	49	23	85
Fine Coal, Heavy Medium	59	21	98

Source: Pittsburgh Mineral and Environmental Technology Internal Documentation

Figure 1: Disciplinary Focus of Coal Biodesulfurization Literature

- ◩ Microbiology
- ⊠ Coal Chemistry
- ■ Process Design
- ☐ Economics/Engineering

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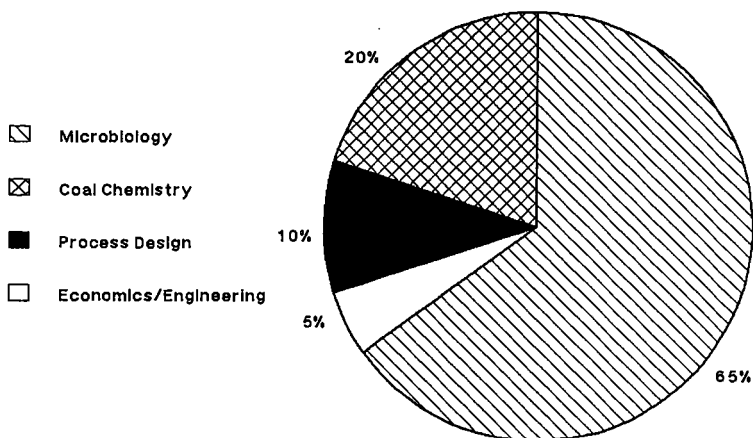
NUMERICAL COMPARISONS BETWEEN THE PYROLYSIS MASS SPECTRA OF TWELVE U.S. COALS
AND THEIR RELATIVE SOLUBILITY IN MICROBIAL CULTURES OR ALKALINE BUFFER

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ABSTRACT

Curie-point pyrolysis mass spectra of twelve low-rank U.S. coals with various
degrees of natural weathering were correlated with the results of biosolubility
screening tests involving six selected microorganisms as well as with a
specially designed alkaline solubility test. The main objective of this study
was to determine which mass spectral characteristics, if any, correlate with the
degree of biosolubility. The results indicate the possible presence of two
biosolubilization trends, a main trend which correlates positively with the
presence of severely oxidized aromatic moieties in the coal and apparently
enables biosolubilization by all six microorganisms, and a second trend which
correlates with relatively high concentrations of acid components and enables
biosolubilization by only two or three of the organisms. The chemical and
biological significance of these trends is not yet completely understood.
Finally, in agreement with previous reports a strong positive correlation was
observed between biosolubility and alkaline solubility.

INTRODUCTION

Few areas of coal science are more challenging and intimidating to analytical
chemists than the study of coal bioliquefaction phenomena. In recent years,
powerful chromatographic and spectroscopic techniques have begun to shed more
light on the structural chemistry of coal. However, the added complexity of
bioconversion phenomena puts coal bioliquefaction processes well out of the
reach of most analytical methods.

A further complicating factor is the lack of standardization with regard to
suitable coal samples, microorganisms and experimental conditions. Although
several coal sample and data banks are in operation within the USA, none of
these banks currently provides the type of relatively strongly weathered, low
rank coals which have been used in nearly all coal bioliquefaction experiments
reported to date. Moreover, many of the various strains of microorganisms used
are not available for general distribution and/or incompletely characterized.
Finally, there is no general agreement as to what constitutes "bioliquefaction"
or how to measure it objectively.

In order to help solve some of these problems Idaho National Engineering
Laboratory has assembled a multidisciplinary task group including researchers at
several different universities. The present communication represents one of
several reports detailing the results of studies carried out during the past
year and primarily aimed at obtaining an improved definition and understanding
of coal bioliquefaction phenomena.

A strong correlation between the bioliquefaction yields and alkaline solubilities of a series of low rank U.S. coals was reported by Quigley et al. [1,2]. Together with the observation that the more "successful" microorganisms tend to produce substantial alkalinity of the culture medium, this suggested that simple acid-base solution processes might play a significant role, especially in strongly acidic, e.g., highly weathered coals. Consequently, one of the key questions in any future coal bioliquefaction studies should be whether proof of covalent bond scission can be obtained or not.

In a separate paper, Ward et al. [3], describe a novel bioliquefaction screening and quantitation method which uses objectively measurable product diffusion zones around standard-sized coal sample "disks" imbedded in solid media. After inoculation with selected microorganisms, characteristic "bioliquefaction profiles" can then be obtained.

Here we report the results of a study of 12 low rank coal samples (representing several different U.S. provinces as well as degrees of natural weathering), each of which was submitted to the new "bioliquefaction profiling" procedure using six different strains of microorganisms. Suitable aliquots of each coal sample were further analyzed by means of Curie-point pyrolysis mass spectrometry, using techniques described by Meuzelaar et al. [4]. Also, alkaline solubilities were determined according to Quigley et al. [2].

Finally, biosolubility profiles, pyrolysis MS profiles and alkaline solubility data were compared by means of canonical correlation analysis, a powerful multivariate statistical analysis approach capable of determining common sources of variance between related data sets.

The primary objective of this study was to search for key structural features of coals (as reflected in the pyrolysis MS profiles), directly associated with bioliquefaction response (as reflected in the "bioliquefaction profiles"). A secondary objective was to obtain information on reaction mechanisms, e.g., with regard to the role of acid-base solubilization vs. covalent bond scission processes.

EXPERIMENTAL

Sample Preparation - Suspensions of each of the 12 coals (5 mg of ~200 mesh coal per ml of spectrograde methanol) were prepared. A 5 ul drop of each suspension (25 ug of coal) was applied to a 610°C Curie-point wire. The methanol was evaporated under continuous rotation of the wire.

Pyrolysis Mass Spectrometry - Py-MS was performed with the MS inlet at 25°C and using low voltage (12 eV) electron ionization. For each Py-MS analysis, the total number of scans was 100 and the total scan time was 23 seconds. Each sample was analyzed in triplicate.

Data Analysis - The Py-MS data were first normalized to 100% total ion intensity. For the normalization of the total ion intensities, mass peak signals with high variance were temporarily excluded. 159 mass peaks with high "characteristicity" (outer variance/inner variance ratio) values were used for factor analysis. Furthermore, these Py-MS data were correlated with biosolubility data and alkaline solubility data by canonical correlation techniques.

Biosolubility - A set of six selected fungal strains, e.g., ACL-12, DML-12, P. Chrysosporium, RWL-40, YML-1 and YML-21, representing a diversity of taxonomic types was used for all biosolubility assays. The general methods used for standardized assays (i.e., the "diffusion zone assay") of coal biosolubility were described elsewhere [3]. In brief, 2-3 mm diameter mounds of sized coals (0.25-0.50 mm) were placed with uniform spacing on the surface of Sabouraud Dextrose 1.5% agar ("SDA" Difco Laboratories, U.S.A.). Medium depth was 5.5 mm in 100 mm diameter pyrex-glass; deep-well culture dishes. Two replicate preparations of five coal units were used for each coal type. Spores or hyphal fragments of each organism were inoculated onto the surface of the nutrient medium after which the cultures were incubated in the dark at 30°C, 70 ± 2% RH for 17 days. Circular zone diameters were measured and averaged to give values for comparisons of degree of biosolubility for each coal.

Alkaline solubility - One half gram of sized coal (0.25 - 0.50 mm) was placed in a 250 ml Erlenmeyer flask containing 50 cc of a pH 8, tris buffer. Flasks were shaken (140 rpm) for 24 hours at 25°C. Supernatants were obtained by filtering through 0.2 micron filters. Where necessary, dilutions were made using fresh TRIS buffer and absorbances at 400 nm measured.

RESULTS AND DISCUSSIONS

The wide range of different coal types included in this study is illustrated in Figure 1. At first sight the low voltage pyrolysis mass spectrum of North Dakota Hagel lignite appears to be fairly characteristic of Northern Great Plains lignites, i.e., dominated by homologous series of dihydroxybenzenes and phenols representative of fossil lignins [5]. However the relatively high peak intensities at m/z 45, 46, 60, 87 point to the presence of carboxylic moieties characteristic of oxidative changes [6]. Since the Hagel lignite was originally obtained from the Penn State Coal bank (see Table 1), these oxidative changes are probably due to long term exposure to ambient air in the laboratory. By comparison, the Mississippi Claiborne lignite (see Figure 1b) shows a much more pronounced homologous series of aliphatic hydrocarbon components. This is again in agreement with earlier Py-MS studies of lignites representing the Gulf Province [5] in which higher relative abundances of aliphatic hydrocarbons were found to correlate with aquatic depositional environments. In that study, longer chain aliphatic hydrocarbon moieties in Gulf Province coals were thought to represent liptinitic macerals primarily derived from algal materials. Finally, the spectrum in Figure 1c, obtained from an Arkansas Lower Hartshorne "bituminous" coal, appears to be totally different. Little or no aromatic compound series are seen whereas aliphatic series are not very distinct either. In fact, this spectrum is almost totally dominated by the (off scale) intensities of small mass peaks in the m/z 28-45 range. In our opinion this coal sample, collected from an old roadcut in highway 7 (see Table 1), has undergone severe chemical deterioration and should perhaps be considered as a humic acid type material rather than a coal. The wide range of structural chemical differences between just three of the twelve coals in our study illustrates the magnitude of the analytical problem.

In order to obtain a more systematic overview of the various chemical components and trends in our data set we performed a factor analysis of all 36 coal spectra (each sample was analyzed in triplicate). A description of the variance described by the first 11 factors with eigenvalue >1.0 is given in Table II whereas a score plot of factors II and III (Figure 2a) was found to display some of the most interesting chemical information. A more complete description of

the information in Factors I and IV will be provided in the final publication of the results. Chemical interpretation of the clustering trends in Figure 2a is facilitated by inspection of the so-called variance diagram plot [7] in Figure 2b which reveals the presence of at least 4 major chemical components. These components can be shown in mass spectral form using a "factor spectrum" technique described by Windig et al. [8]. Inspection of the factor spectra in Figure 3a-d shows two vitrinite-like patterns (component axes B and C in Figure 2b), an aliphatic hydrocarbon pattern (component axis E) and a sulfur compound pattern (component axis F). Comparison with component spectra found in earlier Py-MS studies [5,9] of U.S. lignites and coal maceral fractions, respectively, indicates that, whereas components B and C may be regarded as primarily of terrestrial origin, components E and F can be thought to represent a stronger aquatic influence on the ancient depositional environments involved.

In other words, Factors II and III appear to be primarily correlated with differences in depositional environment. Not unexpectedly, Factor I was found to be dominated by differences in the overall degree of oxidation and/or weathering (factor spectrum of component A not shown here because of space limitation). In contrast to the apparent differences in "reported rank" shown in Table I, no obvious rank dependent influence was observed other than the presence of two distinct, possibly rank dependent, vitrinite-like patterns in components B and C. However, it should be pointed out that the highest rank coal (Lower Hartshorne "bituminous") was severely degraded and that the difference in Py-MS patterns between "lignites" and "subbituminous" coals tend to be relatively minor [10].

After examining the major chemical components and trends in the Py-MS patterns of all twelve coals we undertook a thorough analysis of the "biosolubility" data obtained by systematic studies with a panel of six microorganisms (listed in Table III) using a novel technique developed by one of us (B. Ward) and reported in more detail elsewhere [3]. The biosolubility data shown in Figure 4, can be regarded as a multidimensional biological response surface obtained by measuring the diameter of dark diffusion zones of soluble coal components surrounding small disk-shaped coal pellets imbedded in solid culture media inoculated with selected microoganisms under carefully standardized conditions.

Factor analysis of the biosolubility data shown in Figure 4 reveals that the true dimensionality of the biological response surface is close to 2 with the first two factors explaining more than 97% of the cumulative variance (see Table IV). In other words, no more than two independent sources of variance can be present in the biosolubility data. Both trends are clearly identified in Figures 5a and b. All microorganisms appear to be more or less successful in solubilizing coals 3 and 9 (or, less completely, 8 and 10), whereas only organisms c and e (and to some extent b) succeed in solubilizing coals 2 and 11 (or, less completely, 4 and 12).

This can also be seen in Figure 4 where the two response patterns have been arranged to show the differences in the upper and lower rows, respectively. No significant biosolubilization response was observed for coals 1, 5, 6 and 7. It should be noted here that the two distinct biosolubilization patterns were only observed upon factor analysis of the microbiological data but were not immediately apparent in the foregoing examination of the Py-MS data.

This prompted us to resort to the powerful canonical correlation approach in order to determine which, if any, Py-MS signals correlated with the

biosolubilization trends. Moreover, in view of earlier observations by one of us of a strong correlation between biosolubilization and alkaline solubility under abiotic conditions, we decided to include the alkaline solubility values for all twelve coals in the biosolubilization data set.

Two significant canonical correlation functions were found with correlation coefficients 0.99 and 0.92, respectively. Together these two functions explained 25% of the total variance in the Py-MS data set and 92.4% of the total variance in the combined biosolubilization/alkaline solubility data. Moreover, in agreement with the earlier reports by Quigley et al. [1,2], alkaline solubility was found to correlate strongly (correl. coeff. = 0.93) with biosolubilization. Inspection of the scores and loadings of the two canonical correlation functions, as plotted in Figures 6a and b, reveals the same two trends already noticed in Figures 5a and 5b. This brings up the key question: what is the chemical and biological meaning, if any, of these two trends? In order to help answer this question we calculated the two factor spectra correlating with these trends (a further impression of the most important mass peak contributions can be obtained from the combined loadings plots in Figure 6b). Figures 7a and b show the two mass spectral patterns associated with good biosolubilization (and alkaline solubility) by all microorganisms (Figure 7a, 310° component axis) and with good biosolubilization by microorganisms c and e (Figure 7b, 40° component axis), respectively.

At present, we are unable to provide an unambiguous chemical interpretation of these two trends. However, spectrum 7b is dominated primarily by fatty acid and carboxylic moieties, such as commonly observed in coals weathered in a laboratory environment [6]. Spectrum 7a, on the contrary, exhibits a variety of additional aromatic or polyunsaturated aliphatic signals suggestive of oxidative products of aromatic compounds. One hypothetical explanation could be that Figure 7a represents severe oxidative destruction by natural weathering phenomena enabling a broad range of microorganisms to start solubilizing the coal matrix, e.g., by producing alkaline compounds, as suggested by Quigley et al. [1,2], whereas Figure 7b points to a more extreme accumulation of carboxylic acids, and thus a strongly acidic pH, possibly allowing only some of the microorganisms to produce sufficient alkaline compounds to solubilize the coal.

In line with this interpretation, the alkaline solubility vector in Figure 6b can be seen to be intermediate between the two trends. In other words, both conditions produce an equal degree of solubilization in the presence of an unlimited amount of alkaline buffer. If this explanation is correct, the lower acid concentration in Figure 7a could have been caused by secondary natural phenomena, such as the loss of water soluble acidic constituents through leaching, rather than by a different oxidative mechanisms.

Presently, we are investigating possible other explanations, e.g., involving metal chelating agents or the possible involvement of enzyme-mediated bond breaking mechanisms.

CONCLUSIONS

Multivariate analysis of pyrolysis mass spectra of twelve samples of low rank coals exhibiting different degrees of biosolubility revealed a high level of heterogeneity, apparently associated with differences in rank, depositional environment (e.g., as reflected in maceral composition) and degree of weathering.

Multivariate analysis of "biosolubility profiles", obtained by measuring the relative biosolubility response of twelve coal samples to a panel of six different microorganisms, strongly suggests the presence of at least two biosolubility mechanisms. One of these mechanisms is exhibited by all 6 microorganisms whereas a second mechanism appears to be associated with only 3 of the microorganisms.

Canonical correlation analysis of the pyrolysis mass spectrometery and biosolubility profile data sets produced two canonical variate functions with correl. coeff. 0.99 and 0.92, respectively. Together these functions explain approx. 25% of the total variance in the Py-MS data and 92.4% of the biosolubility data.

Coal samples exhibiting good biosolubility appear to have in common a pronounced oxidative degradation of aromatic structural components, thought to represent vitrinitic and related maceral constituents. Besides increased biosolubility, the most obvious effects of these oxidative changes are: (1) an apparent increase in low MW, oxygen containing pyrolysis products; (2) a notable increase in alkaline solubility; and (3) a markedly reduced abundance of characteristic vitrinitic signals in the pyrolysis mass spectrum.

The observed correlations between the biosolubility and pyrolysis MS profiles further suggest that oxidation of a coal sample (evidenced by strongly increased pyrolysis yields of CO_2 and CO) does not produce biosolubility for all six microoganisms. An additional structural change, characterized by the increased evolution of pyrolytic benzene (presumably derived from benzenecarboxylic acids) may be needed to induce biosolubility for all six microorganisms and thus achieve maximum alkaline solubility levels.

A strong linear correlation (corr. coeff. 0.91) was observed between alkaline solubility (as determined by absorbance of the solution at 400 nm) and the two-dimensional canonical variate space obtained by canonical correlation analysis of the pyrolysis MS and biosolubility profiles. This is in agreement with previous reports by Quigley et al. [1,2].

ACKNOWLEDGEMENTS

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Table I
Origin, Rank and Alkaline Solubility of 12 Coals

Sample #	seam or field (state)	reported rank	description of sampling site	alkaline[a] solubility
1	Coalmont (CO)	subbit	fresh stripmine sample (Kerr Mining CO)	-2.70
2	Coalmont (CO)	subbit	weathered chunks from abandoned mine (near site 1)	-1.09
3	Coalmont (CO)	subbit	erosion gully outcrop in pasture (near site 1)	1.45
4	Kiowa (CO)	lignite	dry gulch outcrop	-1.19
5	Green River (CO)	subbit	weathered chunks from abandoned strip mine (Black Dan, near Hayden)	-2.70
6	Hagel (ND)	lignite	fresh sample (PSOC 1482)	-1.22
7	Clairborne (MS)	lignite	creek bed outcrop near Antioch	-0.78
8	Clairborne (MS)	lignite	ibid, more weathered	-0.36
9	Wilcox (MS)	lignite	I-20 roadcut near Russell	0.91
10	Midway (AL)	lignite	I-20 roadcut between York and Cuba	0.32
11	Lower Hartshorne (AK)	bituminous	Hwy 7 roadcut of excavation near Dardanelle	-0.70
12	Brandon (VT)	lignite	fresh sample stored in H_2O since October, 1984	-0.35

a. Alkaline solubilities (log (absorbance)) were measured at 400 nm

Table II
Factor Analysis Results of Py-MS Data

FACTOR #	EIGENVALUE	%TOTAL VARIANCE	CUMULATIVE VARIANCE
1	88.85	57.86	57.86
2	22.60	14.71	72.57
3	14.99	9.76	82.58
4	8.06	5.25	87.58
5	6.42	4.18	91.77
6	2.85	1.86	93.62
7	2.39	1.56	95.18
8	1.84	1.19	96.38
9	1.64	1.07	97.44
10	1.28	.83	98.28
11	1.06	.69	98.96

Table III
List of Microorganisms and Description of Six Fungal Strains

Bacteria #	Code	Full Name
a	ACL-13	Candida sp.
b	DML-12	Acremonium sp.
c	p. chrysosporium	phanerochaeta chrysosporium
d	RML-40	an unidentified Basidiomycete
e	YML-1	Cunninghamella sp.
f	YML-21	a Cunninghamella - like Hyphomycete

Table IV
Factor Analysis Results of Biosolubility Data

FACTOR	EIGENVALUE	% TOTAL VARIANCE	CUMMULATIVE VARIANCE
1	5.19	86.43	86.43
2	.64	10.59	97.03
3	.14	2.34	99.37
4	.03	.49	99.86
5	.01	.12	99.99
6	.00	.01	100.00
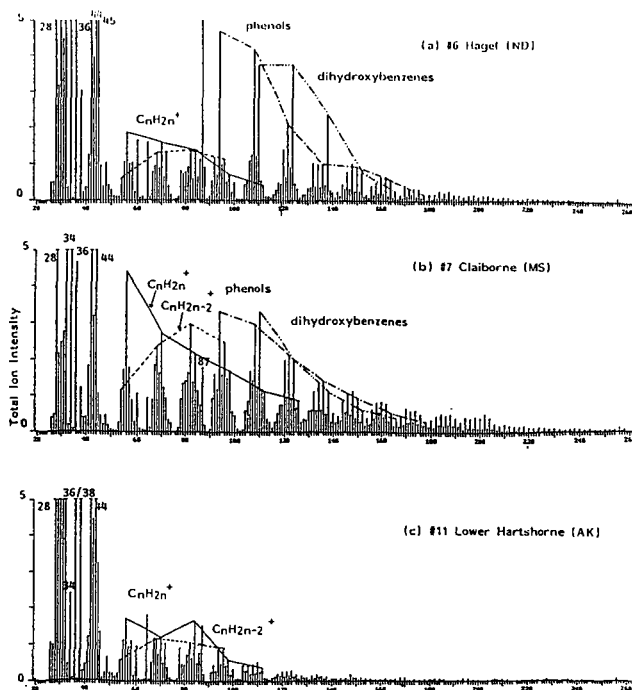
Figure 1. Pyrolysis mass spectra of (a) North Dakota lignite (b) Mississippi Claiborne lignite (c) Arkansas "bituminous" coal.

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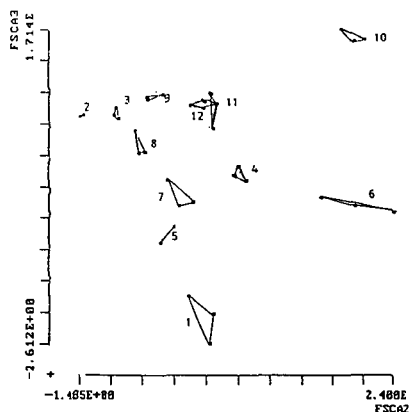
Figure 2a. Score plot of factor 2 vs. factor 3 of MS data.
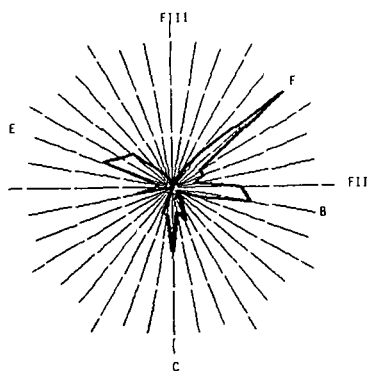
Figure 2b. Variance diagram showing the presence of four major component axes B,C,E,F, in the space spanned by factors II and III.
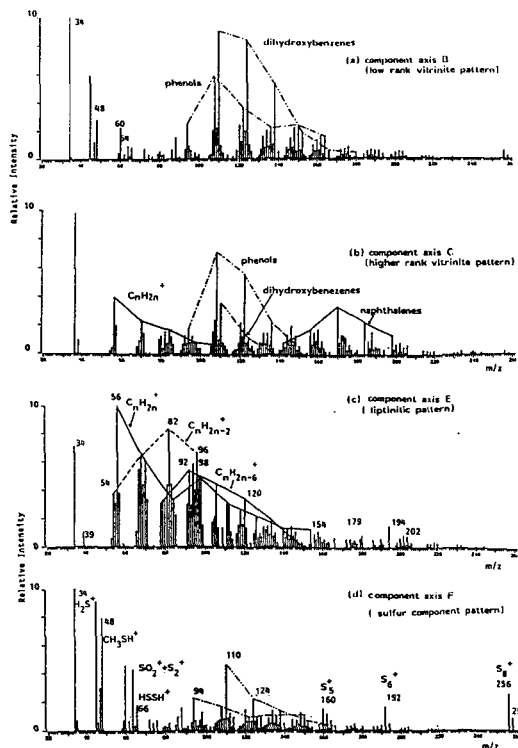
(a) component axis B (low rank vitrinite pattern)

dihydroxybenzenes

phenols

(b) component axis C (higher rank vitrinite pattern)

phenols

$C_nH_{2n}^+$

dihydroxybenzenes

naphthalenes

(c) component axis E (liptinitic pattern)

$C_nH_{2n}^+$

$C_nH_{2n-2}^+$

$C_nH_{2n-6}^+$

(d) component axis F (sulfur component pattern)

H_2S^+

CH_3SH^+

$SO_2^+ + S_2^+$

$HSSH^+$

S_5^+

S_6^+

S_8^+

Figure 3. Comparison of numerically extracted factor spectra of (a) component B (low rank vitrinite pattern), (b) component C (higher rank vitrinite pattern) (c) component E (liptinitic pattern) and (d) component F (sulfur component pattern).
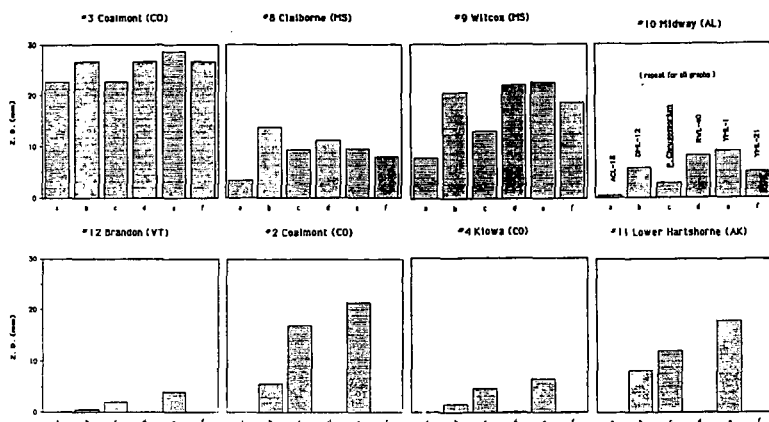
Figure 4. Biosolubility of 8 coals as a function of six microorganisms, expressed by zone diameter (mm). None of the microorganisms shows biosolubility on coals nos. 1, 5, 6 or 7.
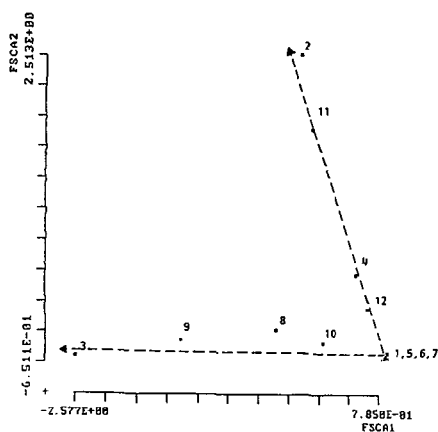
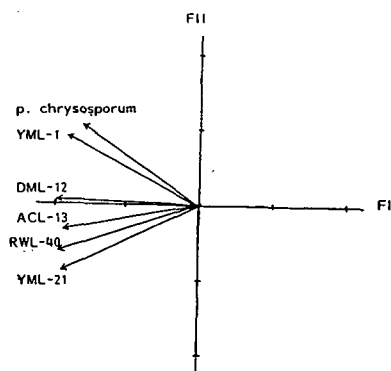Figure 5a. Score plot of factor I vs. factor II of biosolubility data.

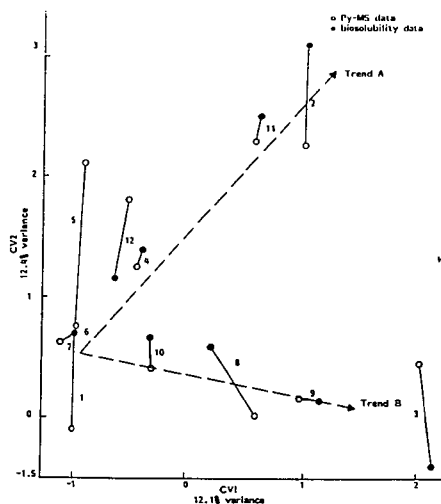Figure 5b. Variance diagram of factor I vs. factor II of biosolubility data.

Figure 6a. Combined score plot of the first two canonical variate function of the Py-MS data and the biosolubility data, respectively.
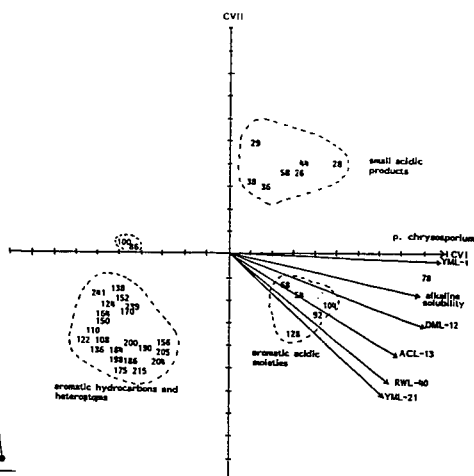
Figure 6b. Loading plot of the mass values and biosolubility parameters contributing most strongly to the first two canonical variate functions.
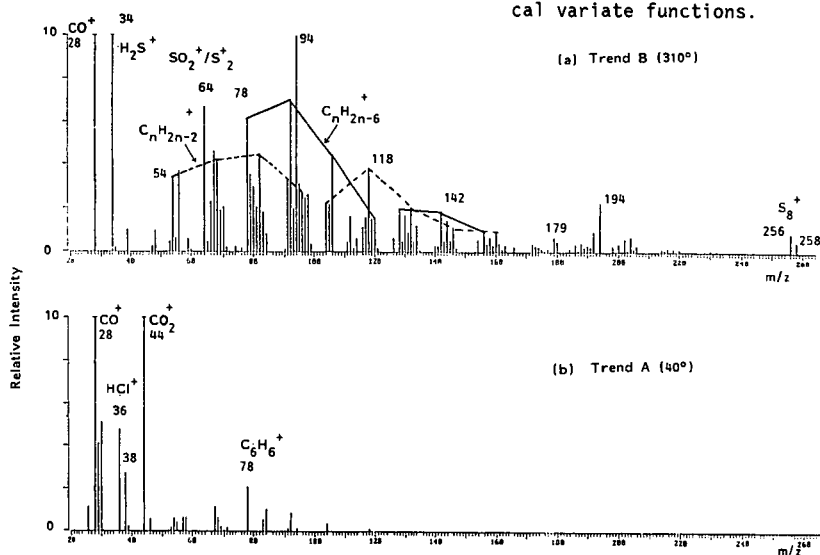

Figure 7. Numerically extracted mass spectra of trends B and A at 310° and 40° in CVI/CVII space, respectively.

ENZYMATIC REMOVAL OF ORGANIC SULFUR FROM COAL

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ABSTRACT

A process in described for the enzymatic (not microbiological) removal of organic sulfur from coal. The process evaluated utilizes ultra ground coal (<50 microns) and froth flotation to first removal pyritic sulfur. In this design conceptual mild alkali oxidation followed by enzymatic treatment is then used to reduce the organic sulfur. A preliminary economic analysis for processing 20,000 metric tons of coal/day is being carried out for reducing 4.5% sulfur content bituminous coal (containing an assumed 50:50 W/W pyritic/organic) to under 1% sulfur in order to meet clean air standards. The background work leading to this conceptual process is encouraging. Experimental work is now proceeding and preliminary results are encouraging.

INTRODUCTION

Background to the Problem of Sulfur in Coal

The use of fossil fuels for power generation is expected to increase, despite diminishing world reserves of high quality crude oil and coal. The demand for low-sulfur and low-nitrogen fossil fuels has been intensified by increasingly stringent regulatory standards for reduced levels of sulfur- and nitrogen-oxides in released atmospheric emissions. Amendments to the 1964 Clean Air Act require not only compliance with emission standards but also a percentage reduction in emissions that otherwise would occur from the burning of untreated fuel, requiring that low-quality fuels be subjected to either pre- or post-combustion desulfurization.

Post-combustion processes, e.g., flue-gas desulfurization, are generally inconvenient, expensive, and definitely limited with respect to the scope of their remedial action. These processes usually ignore other aspects of (sulfur) contamination which are certainly as economically important as emissions control, e.g., corrosion of mills and conveyors due to pyritic sulfur. Pre-combustion cleaning processes, on the other hand, can offer significant advantages over post-combustion processes, such as flue gas desulfurization, because these pre-combustion schemes can eliminate problems associated with both environmental pollution and equipment corrosion.

Traditional pre-combustion processes exploit either physical or chemical methods. Physical methods include flotation or magnetic separation of the pyrite from coal. As compared to chemical methods, these are generally more economical. The drawbacks to these physical methods, however, lie in the limited removal of the overall sulfur and the notable energy losses. Chemical pre-combustion cleaning methods usually employ some sort of oxidizing agent, e.g., ferric salts, chlorine, or ozone, or reducing agent, e.g., solvent-hydrogen mixtures. In contrast to physical methods, chemical methods can be reasonably effective in removal of some of the organic sulfur along with the pyrite. Some of the disadvantages which have an impact on the efficiencies associated with chemical

cleaning operations include corrosion problems resulting from the use of high concentrations of the chemical agents, the energy intensiveness of the high temperature/high pressure operations, and the costliness of reagent recovery.

Thus, there is a need for methods of coal desulfurization which improve upon these traditional process. Of particular need is a method for removal of organic sulfur, a contaminant which often is only secondarily removed. This paper focuses on a processing concept for the resolution of that need. Drawing upon the experiences derived from the aforementioned types of cleaning operations, the technical feasibility of a novel concept is considered within the context of process efficiency. Reagent costs, recovery operations, waste products, and operating conditions are also under consideration in this new plan for coal beneficiation while the technical concepts are researched. Development goals and research goals are of coincident interest, as is necessary for programs addressing new cleaning processes.

Background to Biodesulfurization

An obvious means to the circumvention of some of the cost problems found in traditional coal cleaning processes lies in the use of biological methods because of the inherently lower capital and operating costs. Indeed, microbial coal desulfurization already has a history. Early interests in this field focused upon microorganisms for pyritic sulfur removal; these microorganisms were "naturally" suited to this task having themselves adapted to sulfur utilization in mine waters (e.g., as with Thiobacillus) or sulfur springs (e.g., as with Sulfolobus). However, while interesting from the standpoint of process-type manipulations, these microorganisms do nothing for organic sulfur removal. Thus, if substantial sulfur removal is of interest, biological methods must do more than remove only the inorganic sulfur.

Several more recent efforts have focused upon the adaptation and use of various strains of microorganisms to act upon and remove the organic sulfur (e.g., those projects at the Atlantic Research Corporation[1,2], The Institute of Gas Technology[3], and the University of Georgia[4]. These "coal bugs" are becoming well-known and have been shown to have "appetites" for organic sulfur. Oxidation of sulfur for removal as organic sulfate has been the most commonly demonstrated mechanism for microbial removal of organic sulfur; as a result, some of the organic, i.e., the energy, content is often lost as part of the sulfate. While encouraging demonstrations of the feasibility of organic sulfur removal via biological means have been shown, these projects by no means represent optimized solutions. Microbial efficiencies and process operations await improvements. These works continue, for the most part, in that vein while additional lessons in coal cleaning are made available for further conceptual developments of the sort being exploited, as described herein.

A consideration in these microbially-based cleaning operations lies in the potential for some degree of process sensitivity. That is, because these operations exploit live organisms, there is concern as to their maintenance as both viable and selective populations. While ample precedent exists for microbially-based treatment systems, e.g., wastewater treatment, there are recognized sensitivities implicit in the use of live systems, such as mutational problems, reactor upsets, substrate variations, etc. A possible solution may lie in the use of an enzyme-based cleaning process, that is, a process which solely uses the "active extract" of the microbial process to effect desulfurization[5]. The concept of enzymatic removal of sulfur from coal is discussed in the following.

Enzymes for Removal of Organic Sulfur From Coal

An enzymatic (not microbiological) process for organic sulfur removal from coal would first require the identification, characterization and production of an enzyme known to cleave sulfur from a representative polymer. These enzymes would then be immobilized or "tethered" on a standard packing material such as is used to support conventional catalytic materials in the bioprocess industries (the enzymes may be in solution, as discussed later, but immobilized enzymes are used in this explanation.) Upon passing the coal/water slurry through the packing material, the immobilized enzymes tethered on the packing would cleave the organic sulfur, acting as a type of biocatalyst. Coal particles, free of organic sulfur, would then be recovered; sulfur could also be readily recovered.

With this introduction, some of the pertinent technical background is given in the following section.

TECHNICAL BACKGROUND
Overview

The subject of sulfur removal from fossil fuels by biologically based means has been receiving increasing attention in recent years(6). This is due, in part, to the enhanced ability of controlling the genetic make-up of various organisms, but also to the possibility of selectively removing the objectionable sulfur compounds at the front-end of a power generation process and without affecting the hydrocarbon structure (hence, the thermal value) of fuel. Other advantages include mild operating conditions, possible use of natural earth formations as reactor systems, and possible longer biocatalyst life under concentrated sulfur and heavy metal conditions observed in refining and cracking operations.

Pyrite Removal

Sulfur content of coal has been reported to be at least 4.5%, approximately equally distributed between organic and inorganic compounds. The latter, predominantly in the form of pyrite, have been investigated recently, with regards to their dissolution potential through the mediation of soil acidophilic bacterial. A large number of such organisms have been isolated to date, the most important among them being members of the genera of Thiobacillus and Sulfolobus. Extensive studies with such organisms, in pure as well as in mixed cultures, demonstrated the feasibility of oxidative dissolution of the pyrite sulfur from coal(7,8,9). Dissolution kinetics were found to be first order with respect to the specific surface area of the coal, and, based on these preliminary kinetic data, a feasibility assessment of a coal water slurry pipeline was carried out for the simultaneous transport and pyritic sulfur removal from the coal.

Organic Sulfur Removal

The removal of organic sulfur from coal is a significantly more demanding task than pyritic sulfur removal mainly because of two reasons. Foremost, organic sulfur is deeply embedded in the coal matrix requiring a very fine pulverization of coal in order to expose the sulfur compounds to the action of chemicals or catalysts. Second, the primary sulfur compounds are far more resistant to degradation or assimilation by organic or biological catalysts and often poisonous or toxic to their activity.

The possibility of using microorganisms for organic sulfur removal from coal

has been examined in the past forty years. The patent literature is rather imaginary and inconclusive. The majority of these studies were carried out with model compounds representative of the three types of organic compounds that the approximately 195 sulfur substances in coal can be classified into, namely, A) sulfides, B) thioles, and C) thiophenes(10). In particular, due to the relative ease of removing aliphatic sulfides and other sulfur compounds of low boiling point by simple chemical means, most of the attention has been focused on aromatic sulfur compounds such as thiophenes, especially benzothiophene (BT) and dibenzothiophene (DBT)(10). Attempts to isolate microorganisms capable of degrading simple thiophene aerobically have been unsuccessful(11). However, microbial degradation of the nucleus of substituted thiophenes has been demonstrated(12). Other studies have addressed the possibility of degrading BT and DBT. For example, a mixed culture of Arthrobacter sp. and Pseudomonas sp. was shown to be able to grow on DBT and residue oil with products being cellular mass and inorganic sulfate(13). DBT could also be converted to water soluble oxygenated derivatives which accumulated during metabolism, but the sulfur in the thiophene nucleus remained untouched by this action(14). Several other studies have confirmed these findings showing that BT, DBT, and related organic sulfur compounds can be converted into soluble sulfur compounds through the use of pure cultures or microbial consortia(15-17).

There are, however, some serious problems associated with the use of living organisms in considering large scale organic sulfur removal from coal. First, the conditions which are optimal for the growth and activity of such organisms are usually incompatible with the concept of the envisioned large-scale process or require expensive additives so that they can be maintained for extended periods of time. Second, objectionable amounts of growth factors and carbon sources are needed which could cause insurmountable economic difficulties. Third, toxic by- products or other compounds in a realistic process environment could be the source of severe poisoning problems that render the microorganisms incapable of performing the tasks for which they were isolated. Finally, culture stability is always a serious concern especially in open, continuous flow ecosystems. For this reason, the concept considered here is a combination of chemical pretreatment with an enzymatic process which could be a viable alternative to the above mentioned possibilities.

Enzymatic Desulfurization Process

It is assumed that it is feasible and economical to pulverize coal down to 10 to 50 micron fine particles so that most of the organic sulfur compounds become either exposed to or reachable by the chemical reagents involved in the pretreatment and the subsequent enzymatic process. If this is not so, significant carbon oxidation may take place before the sulfur compounds can be acted upon by the oxidizing agents.

The coal particles are mixed with water for the preparation of a coal/water slurry (available technology) which is subsequently subjected to a chemical oxidation pretreatment with a hot alkaline reagent. The objective is to partially oxidize the maximum possible amount of the organic sulfur compounds to sulfate. Full oxidation to SO_4 is obviously undesirable and so are many destructive effects of the oxidizing agent on the carbon of the coal matrix. There is clearly an optimum for the degree of oxidation, and this can be achieved by varying the temperature, strength of alkali, and duration of pretreatment, followed by titration for the fraction of overall sulfur converted to sulfate for each combination of operating conditions.

The next step is to remove the sulfates from the coal particles. One way to selectively accomplish this is by contacting the coal/water slurry with a sulfatase enzyme solution. Enzymes are commercially available that have the property of catalyzing the dissolution of sulfur compounds by cleaving the sulfate anions from the aliphatic aromatic backbone. The resulting sulfate solution can be further treated with lime to finally contain sulfur in the form of calcium sulfate solids.

Concept Presented

The present work addresses the feasibility of organic sulfur removal from coal via select enzymatic methods. These methods focus upon the stepwise biochemical transformation of the coal matrix for extraction of the organic sulfur as sulfur oxides such as sulfate. These transformations proceed via enzymatic pretreatment of the coal to both (bio)chemically oxidize the available organic sulfur and (bio)physically "loosen" the matrix followed by enzymatic cleavage about the pretreated sulfur moieties.

Thus, this concept followed from the basic demonstrations of microbial desulfurization in that technical sense which supports oxidative methods for removal of organic sulfur. However, this concept focused on a unique approach to circumvention of the process inefficiencies associated with microbial desulfurization methods by testing the use of only the "active' fractions of microbial populations, i.e., the enzymes, to effect the necessary coal chemistry for sulfur transformations and removal. This evaluation was conducted within the context of considering a feasible process template for an enzymatic coal cleaning operation. A brief discussion of the enzymatic removal of organic sulfur from coal, the concept on which our concept is based, is as follows.

ENZYMATIC COAL BIODESULFURIZATION

Rationale For Processing Concept

The intricacies of coal structures and their chemistries are the subject of continuing study. However, some of the basic elements of coalification and depolymerization chemistries can be considered. Coal is formed via dehydration processes and coal is broken down via combinations of hydrolytic, oxidation, and other displacement chemistries. The understanding of these types of chemical transformations in coals forms the basis for various processes addressing coal upgrading or beneficiation via traditional methods, e.g., liquefaction or chemical desulfurization.

Microorganisms which have been shown capable of interacting with coal also take advantage of these coal chemistries. Basically, microbes establish an interaction with the coal because the coal has something which the microbe can use or, at least, tolerate. The microorganism requires its "balanced diet" for growth and survival. If given coal as the mainstay of that "diet", then it has to acclimate its biological machinery in such a way so as to be able to utilize the coal. This means that the microorganism's consortia of available reagents (e.g., water) and catalysts (i.e., enzymes) must go to work to selectively extract and/or cleave the nutrients it needs, and, in doing so, this micro-organism uses established chemistries. There is no reason to suggest that, in these instances, coal is extracted or cleaved in a manner any different than those demonstrated via conventional chemical processing. Indeed, some of the early work which has been done on the mechanisms of microbially-mediated coal

processing shows (through product analysis) that this is certainly so. Studies
of microbial liquefaction, for example, demonstrate oxidation and hydrolysis of
the coal matrix.

Current knowledge of microbial desulfurization processes lends further
support to the cited mechanism for coal breakdown. Those microbial processes
specifically intended for organic sulfur removal from coal implicate oxidation of
the carbon-sulfur linkages to yield sulfur oxides (in most cases, sulfates) which
then are susceptible to a hydrolysis to liberate the sulfur oxides from the
remainder of the coal matrix. There have been, however, some serious problems
associated with the use of these living organisms in large-scale organic sulfur
removal from coal. First, the conditions which are optimal for the growth and
activity of such organisms are usually incompatible with the concept of the
envisioned large-scale process or required expensive additives so that they can
be maintained for extended periods of time. Second, objectionable amounts of the
growth factors and carbon sources are needed which could eventually cause
insurmountable economic difficulty. Third, toxic by-products or other compounds
found in a realistic process environment could be the source of sever poisoning
problems that render the microorganisms incapable of performing the
desulfurization task for which they were isolated. Finally, culture stability is
always a serious concern particularly in an open, continuous-flow ecosystem.

Given microbial ability to effect the coal chemistries necessary for
desulfurization, but also given the concerns of a "living" processing system, our
team early considered an alternative biologically-based desulfurization scheme.
In this alternative concept was seen the advantages of bioprocessing in perhaps
an eventually more efficient manner. Specifically, the concept investigated has
focused on the sole use of the "active extracts" of the microbes with the
consideration that the problems associated with reactor upset can be minimized.
The concept subject to the investigation is an enzymatic desulfurization process.

While enzymatic processes have been proven in large-scale industrial
applications, it is important to note that their use in coal cleaning is not
merely a transfer of technology to a new process substrate. Specific enzymatic
action about the organic linkages of interest, as well as the efficiencies of any
demonstrated action, must be investigated. The issues of enzymatic accessibility
must also be addressed. In addition, the constraints imposed by a solid
substrate must be incorporated into design consideration, particularly if the use
of raw coal is the major interest. Thus, both scientific and engineering
feasibilities are important components to process development.

Processing Concept

Overall, the present concept is a desulfurization system that derives its
processing reagents from biological sources. In contrast to chemical operations,
processing catalysts (i.e., enzymes) are of natural origin thus, potentially,
processing may be more benign. In contrast to microbial operations, the active
agents are molecularly discrete; thus, potentially, processing may be less
sensitive to the reactor upsets associated with "living" microbial systems.

In this concept enzymes are being used as processing tools in an effort to
affect the removal of the organic sulfur from coal. The coal chemistries are
those of oxidation and hydrolysis. "Oxidases" are being used to catalyze the
oxidation about the sulfur bond; "hydrolases" are being used to catalyze the
hydrolysis about the prepared sulfur. In other words, biochemicals are being

investigated for their potential to direct the chemistry of desulfurization thus leading to a potentially more efficient cleaning process. The overall objective is to investigate the feasibility of a practical, bioreactor-based enzymatic desulfurization process specifically intended for organic sulfur removal from coal.

Technical Considerations

The basic premise of our concept for enzymatic desulfurization of coal is that the use of specific hydrolases will reduce the organic sulfur content. For example, the reaction to remove organic sulfur may use one type of hydrolase, namely an "arylsulfatase". That is, in the presence of the arylsulfatase enzyme, the hydrolysis of organic sulfate compounds to organic phenols is catalyzed. The sulfur is effectively isolated from the organic starting components and is retrievable as water-soluble sulfate. Aside from the issues of biocatalyst selectivity and operating efficiency, the use of an arylsulfatase for organic sulfate removal is fairly straightforward.

The complexity which arises from the actual exploitation of arylsulfatases or sulfur removal lies in the consideration of the forms of sulfur in coal. The three major forms of organic sulfur in coal are: (1) thiophenes (approximately 65% of the total organic sulfur), (2) sulfides (approximately 25%), and (3) thiols (approximately 10%). Very little of the accountable sulfur is presumed to be in the form of organic sulfur oxides, such as sulfate. However, it is possible to survey these available sulfur forms and propose their conversion to oxides for removal via hydrolase, e.g., sulfatase, enzymes. Specifically, it is our concept to oxidatively pretreat coal for conversion of as much of the organic sulfur to oxides, such as sulfate, then effect the actual desulfurization via enzymatic hydrolysis of the sulfur oxide.

Thus, our concept for enzymatic desulfurization of coal presently organizes the processing effort into two areas: (1) oxidative pretreatment, and (2) actual desulfurization. The former has as its goal the oxidation about the sulfur moiety and includes investigation of both chemical and enzymatic methods. The latter has as its goal the actual removal of the prepared sulfur from the organic matrix and focuses on enzymatic methods only. The results in each area are intended for use in a reasonable process model, the template of which has been prepared.

Acknowledgement

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SOLUBILIZATION OF LEONARDITE BY WHITE-ROT FUNGI GROWN IN STATIONARY AND SHAKE FLASKS

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INTRODUCTION

Oxidized coals, including a naturally oxidized lignite identified as leonardite, are solubilized and sometimes degraded further by a variety of fungi and bacteria. Evidence for biosolubilization of coal was first presented by Fakoussa (1), and Cohen and Gabriele (2). Subsequent studies concentrated on screening organisms (3), characterization of the product (4), and determination of the biochemical mechanisms. Mechanisms of biosolubilization are poorly known and may vary with the species used and the media. There is evidence for both enzymatic degradation (5,6) and alkaline solubilization (7,8).

The objective of this study was to discover critical factors in solubilization and biosolubilization mechanisms ,by testing a variety of growth media, growth conditions, and fungi. Lignin-degrading species were emphasized because of similarities between the structures in lignin and in low-rank coals. The results indicate that during idiophase (secondary metabolism), the fungi produce alkaline materials that solubilize leonardite.

METHODS

Phanerochaete chrysosporium BKM-F 1767 was obtained from Dr. T.K. Kirk of the U.S.D.A. Forest Products Laboratory; Trametes versicolor (ATCC 12679), from the American Type Culture collection; and Candida ML-13, from Dr. Bailey Ward of the University of Mississippi. Others were isolated at PETC. All were maintained on Sabouraud maltose agar (SMA) or broth at 35°C and 90%-98% humidity. Malt agar was frequently used to promote spore production in P. chrysosporium. P. chrysosporium was inoculated using filtered conidial suspensions (A_{650} = 0.5/cm); other species were inoculated using fungal mats ground in a blender.

To solubilize leonardite on fungal mats, small (1-2 mm) pieces of sterile leonardite were placed on 1- to 3-week-old agar cultures. The pH of the agar was monitored by the use of puncture and surface pH probes, and the black drops of solubilized coal were sampled for pH and other analyses. Elemental analyses were performed on 6-day-old mats and associated agar to study nutrient depletion.

Four broths were tested for their characteristics in supporting fungal growth and promoting solubilization: Sabouraud maltose broth (SMB), nutrient broth (NB), tryptic soy broth (TSB), and a defined broth (DB) (9). Leonardite, a naturally oxidized lignite, was provided by American Colloid Co., Reeder, North Dakota.

Fungi were grown in 125-mL flasks (20 mL of broth) and 250-mL flasks (40 mL of broth). Roughly 40 flasks were set out at one time under the same growth conditions. From this set, duplicate samples were selected once or twice per week. They were filtered (1.0 μ-pore glass fiber) and analyzed for fungal dry weight (using tared filter paper), pH, and absorbance (A_{450}). A routine test was developed to assay the activity of cell-free broth for solubilization using both gravimetric and spectroscopic measures. Unless otherwise stated, leonardite [0.3% (wt/vol), <100-mesh] was added to the cell-free broth, and the mixture was shaken on a wrist shaker for 1 hr. The treated coal was filtered with a tared glass fiber filter (1-μ-pore), washed, dried, and weighed to gravimetrically determine the percentage of coal solubilized. The filtrate was analyzed for pH. The optical density at 450 nm (A_{450}) was measured, using diluted samples when appropriate, to obtain the spectrophotometric determination of the relative amount of coal solubilized. When plotted against each other, the two measures were correlated but showed considerable scatter (R^2=0.820-0.854).

To determine the effect of shake time on solubilization, replicates of cell-free filtrates were shaken and then filtered, and A_{450} of the filtrates were measured at 15-min intervals over the course of 1 hr. The SMB treated with P. chrysosporium was boiled for 1 min or heated at 90°C for 1 hr to determine if denaturation of basic proteins reduced solubilization. Protein concentrations of cell-free extracts were determined with the Bradford test (10).

RESULTS

Solubilization on Agar

Phanerochaete chrysosporium, Trametes versicolor, Candida SL13, Rhizopus arrhizus, and Geosmithia argulus all solubilized 1-2 mm leonardite chunks when grown on SMA at pH over 7 (Fig. 1). Solubilization was evidenced by the appearance of black liquid surrounding the coal within 48 hours after the coals were added to 1- to 3-week cultures. Two possible conversion mechanisms were investigated in the case of P. chrysosporium: degradation by the ligninase enzymes and solubilization by alkaline products. Ligninase is produced by the organism when nitrogen available for growth is nearly exhausted (9). Neither nitrogen nor two other growth essentials, phosphorus and carbon, were depleted in 6-day cultures (Table 1). Thus, conditions for ligninase production are not evident, even though conversion of coal was observed. Furthermore, ligninase is most active at low pH (9). Solubilization appeared to result from production of alkaline materials. This phenomenon was investigated in more detail using suspension cultures.

Solubilization by Fresh Broth

Results of shaking fresh broth with leonardite for 1 hr are shown in Fig. 2. The SMB and NB were preferred for use as growth media in fungal experiments. The TSB was eliminated because of high levels of solubilization in absence of fungi. Later experiments showed that only low levels of biosolubilization were

observed with extracellular fluid harvested from fungi grown in DB. All bio-solubilization data presented below have been corrected for the absorbance due to the medium alone.

Solubilization by Harvested Cell-Free Broth

Particle size was expected to affect solubilization because of the larger surface area presented by the smaller particles. The A_{450} did increase as expected (Table 2) but not greatly within the 60- to 300-mesh range. Results for the standard size used in these studies (<100 mesh) were most similar to those for the 200-300 mesh.

The time needed to solubilize leonardite in cell-free broth harvested from an active fungal growth was studied. The absorbance was determined after shaking and reading A_{450} at 15-min periods for 1 hr (Table 3). The solubilization rate is most rapid during the first 15 min, and there is little increase in A_{450} during the next 45 min or for several hours (data not shown). Thus, the solubilizing agent works quickly, and the 1-hr shake time used under standard conditions is more than adequate.

Leonardite is an acidic material. Addition of leonardite to the harvested growth media reduces its pH and adversely affects its solubilization capacity. As may be seen in Table 4, the percentage of leonardite solubilized as measured by the gravimetric method steadily declines as the leonardite loading is increased. The A_{450} passes through a maximum and begins to decline as the pH is progressively reduced. Thus, the pH ultimately reached by a medium must be taken into account when comparisons of activity are made. The lowest coal loading (0.3 wt%) was chosen to minimize the influence of coal acidity.

A large set of flasks (~40) were set out under the same conditions as described under Methods. Duplicate flasks were selected and their cultures were tested regularly for solubilization of leonardite. The relationship of biomass, pH, percent solubilization, and A_{450} with P. chrysosporium in SMB is shown in Fig. 3. In this example, solubilization and A_{450} parallel the change in pH. Solubilization peaked during secondary metabolism and declined during the autolysis stage. Maximum absorbance occurred at 20-25 days in other runs with SMB cultures of P. chrysosporium.

The time to reach maximum absorbance could be reduced by continuous gentle agitation of the cultures at 50 rpm (Fig. 4). The highest absorbance occurred before the pH reached a peak. Agitation at 200 rpm considerably reduced solubilizing ability, although the pH of broth was similar to that obtained with more gentle agitation. Absorbance levels similar to those shown in Fig. 3 were found with filtrates of NB cultures of P. chrysosporium and SMB cultures of Trametes versicolor (data not shown). Absorbance peaked at 20-45 days.

Extracellular proteins are likely agents of biosolubilization. The Bradford test (10) was used to determine protein concentration in harvested broth as a function of growth time over a 35-day period; protein content increased gradually to between 100 and 200 µg/mL. To determine whether denaturing of the proteins could reduce solubilization activity, samples of active broth from P. chrysosporium grown in SMB were boiled for 1 min or heated at 90°C for 1 hr. Boiling for 1 min resulted in decreases in A_{450} of 60% in one case and 33% in another. Heating a third broth at 90°C for 1 hr reduced A_{450} from 2.5 to 0.2

(92%). These reductions are evidence that biochemical activity, possibly due to proteins, is lost on heating.

DISCUSSION

Solubilization of untreated leonardite was shown to occur with fungi grown on agar and neat filtrates of suspension cultures. Biosolubilization of leonardite or oxidized coals has been attributed to enzymes (2,5), alkaline materials (7), and basic polypeptides or polyamines (8). Alkaline materials can neutralize acidic functional groups and render the coal soluble. In the present study, biochemical activity was only observed after the agar or broth pH increased above pH ~7. However, elevated pH was not sufficient for observation of peak biochemical activity because the most active broths appeared for various periods after pH had increased to a plateau. The protein content of the broths increases with growth time but with a pattern different from that for the increase in pH. Heating of the active broths decreased activity, as would be expected if an agent of biosolubilization was protein subject to loss of activity through denaturation. However, the operation of the agent at elevated pH, and the appearance of bioactivity before nutrient nitrogen starvation, are facts inconsistent with ligninases, such as described by Tien and Kirk (9), being responsible agents. Apparently the elevated pH is a condition that enables peak biosolubilization to be observed; this activity is apparently due to heat-sensitive proteins that may act on leonardite within 15 minutes to bring about solubilization in a standard activity test.

ACKNOWLEDGEMENTS

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Table 1. Elemental Composition (mg) of Phanerochaete chrysosporium at 6-Days Old, and Decrease of elements in Sabouraud Maltose Agar Growth Medium

	Fungus	Elements in agar, day 0	Elements in agar, day 6	Decrease of elements in agar
C	248 mg	1150 mg	854 mg	296 mg
H	36	102	102	0
O	162	749	695	54
N	28	63	46	17
S	3	12	11	1
P	5	<=1	1	0
Dry Weight	482 mg	2077 mg	1709 mg	

Table 2. Effect of Particle Size on Solubilization of Leonardite by Filtrates from SMB Cultures of Phanerochaete chrysosporium.

Mesh Size	A_{450}
60 - 100	0.834
100 - 200	1.110
200 - 300	1.122
<100	1.154

Table 3. Effects of Shake Time on Rate of Solubilization (A_{450})
of Leonardite per 15 min and 1 hr by Fungus-Treated Broths.

pH	Absorbance (A_{450}) 15 min	Absorbance (A_{450}) 60 min	Species
7.9	1.80	2.40	TV
7.4	4.00	4.60	PC
7.4	5.10	5.10	TV
5.9	0.25	0.37	TV

Note: TV = Trametes versicolor
       PC = Phanerochaete chrysosporium

Table 4. Effect of Leonardite Concentration on Solubilization with
P. chrysosporium Culture at Initial pH 7.9 and Initial A_{450} = 1.3.

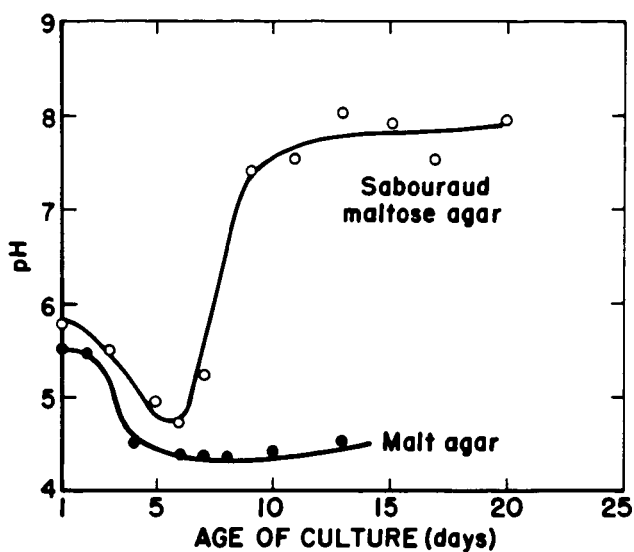| Concentration % (wt/vol)	A_{450}	% Solubilization	Final pH
0.3	4.0	43	7.9
1.0	5.1	11	7.5
3.0	3.9	2	6.2
10.0	1.3	1	4.6

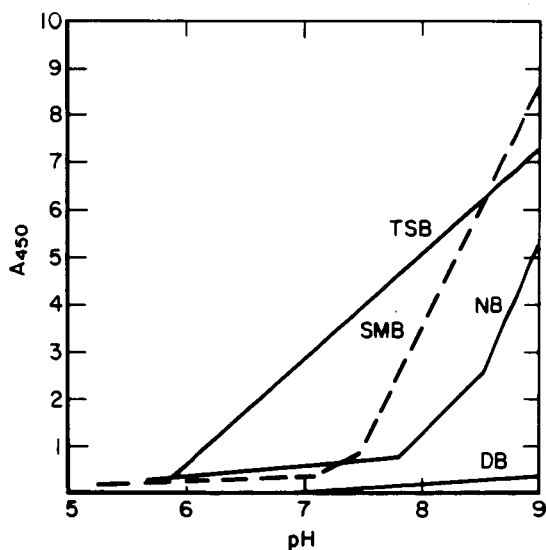Figure 1. Change in pH of agar associated with growth *P. chrysosporium.*

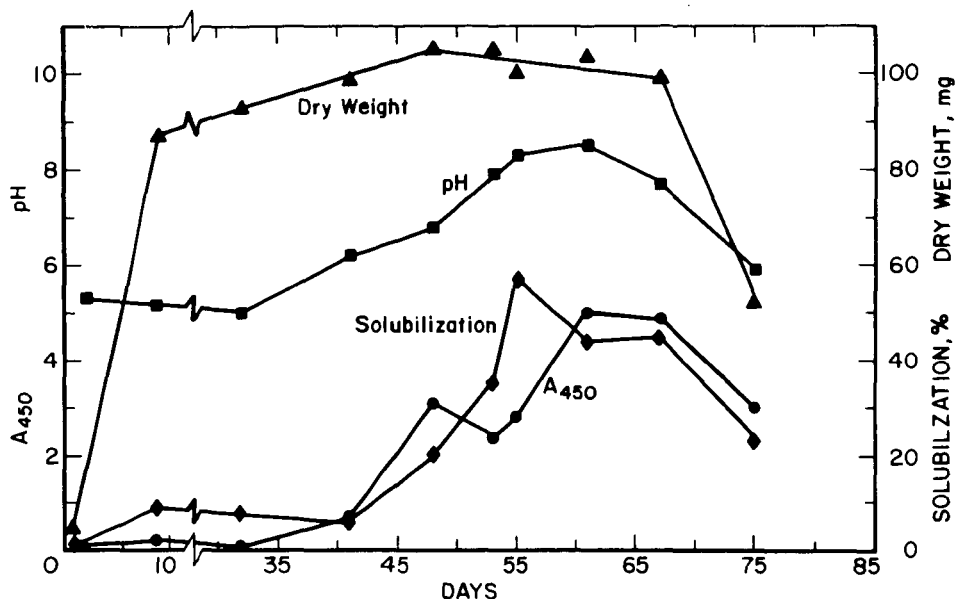Figure 2. Solubilization of 0.3% leonardite after shaking for one hour with fresh broths.

Figure 3. Relationship of dry weight, pH, percent solubilization and A450 from solubilization of leonardite by filtrates from SMB cultures of *P. chrysosporium.*
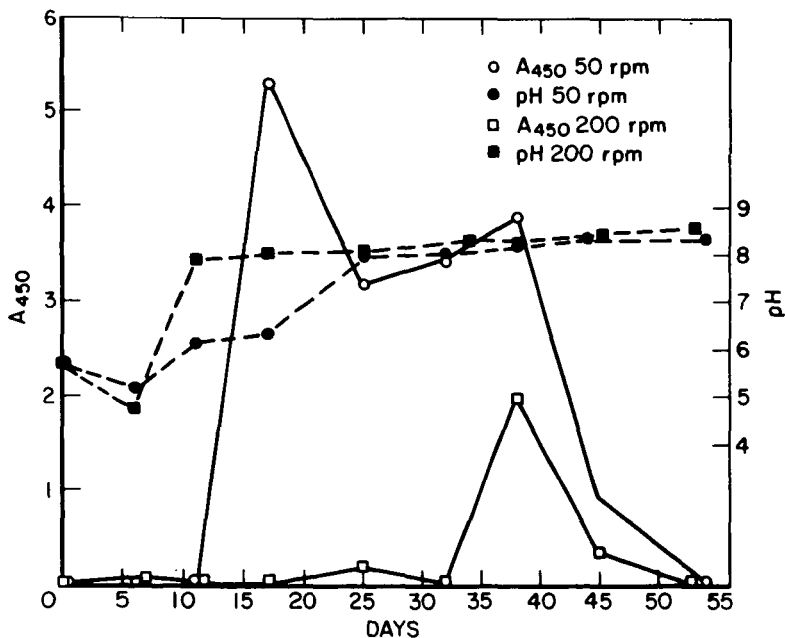

Figure 4. Solubilization of leonardite by filtrates from SMB cultures of *P. chrysosporium* at 50 rpm and 200 rpm.

KINETIC ELEMENTS IN THERMOPHILIC-MICROBIAL
LEACHING OF SULFUR FROM COAL

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ABSTRACT
A mechanism found suitable for pyritic sulfur removal from coal by
mesophilic organisms has been modified and evaluated for the same reaction
by the thermophile: Sulfolobus acidocaldarius. The mechanism as modified
includes direct and indirect mechanistic elements whose magnitudes were
determined experimentally at leaching conditions with and without microbes.
Chemical leaching of pyrite by Fe(III) ions and the chemical oxidation of
Fe(II) to Fe(III) were found to contribute significantly to overall
conversions at thermophilic temperature (72 ^{0}C) but not at mesophilic
temperature (25-37 ^{0}C). The model thus constructed fit overall sulfur
leaching data well after suitable assumptions had been made for extent of
cell attachment; gas (oxygen) transfer rates, sulfur accessibility, and
sulfate reprecipitation rates. The resulting equation is useful for process
sumulation, control and design. Further work is needed to define additional
sulfur leaching effects.

INTRODUCTION
The Sulfolobus acidocaldarius microbe is thermophilic and acidophilic
with optimum growth at temperatures between 70-75 ^{0}C and a pH 2-3[1]. The
microorganism can grow autotrophically on elemental sulfur, mineral
sulfides, and ferrous ions, while it can also grow on glucose and yeast
extract[1,2]. Recent reports claimed the oxidation of dibenzothiophene (DBT)
and organic sulfur in coal by this organism[3,4].
The thermophilic organism S. acidocaldarius has some advantages over
the widely used mesophile Thiobacillus ferrooxidans for microbial coal
desulfurization[5-7]. Its extremely thermophilic, acidophilic, aerobic and
autotrophic character make it resistant to contamination. The rates of
chemical reaction between ferric iron ions and pyrite are higher at high
temperature, which benefits the process of sulfur removal. The solubility of
some mineral sulfates is higher at low pH and high temperature, which
reduces reprecipitation in a process for sulfur reduction. Finally,
microbial ability to remove organic sulfur is very important.
Mathematical models have been proposed to describe the kinetics of
microbial coal desulfurization[8,9]. The model designed for mesophiles
neglected chemical reactions which occurred in parallel with biological
reactions in coal-water slurry system. The model proposed in this report
considers both chemical and biological reactions, as more realistic for
sulfur bioleaching at elevated temperatures. Microbial oxidation of ferrous
ions and of sulfur in coal were studied to provide particular values for the
model parameters.

MATERIALS AND METHODS
Culture methods
The medium for microbial growth was a basal salts solution and an energy
source with or without supplements. The basal salts solution had the

following composition (g/l): $(NH_4)_2SO_4$, 1.3; KH_2PO_4, 0.28; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2 \cdot 2H_2O$, 0.07; in 1 liter of tap water. The energy source can be elemental sulfur, ferrous ions or sulfur in coal. Sometimes yeast extract was a supplement. The pH of the medium was adjusted to 2.0 with 3.0 N H_2SO_4.

The original culture of S. acidocaldarius (ATCC 53230) from Dr. Brierley at the New Mexico Institute of Mining and Technology and transfers from Dr. Kargi at Lehigh University were used throughout the investigation.

Eight hundred ml. of the medium were placed in a three necked, round bottomed flask equipped with a condenser. The system was autoclaved at 121°C for 15 min and then inoculated with a culture of S. acidocaldarius. Microbial cells were grown and harvested from a medium where elemental sulfur was the energy source 3-5 days after inoculation. The culture was first centrifuged at 1000x g for 5 min to remove the sulfur particles. Then, the cell solution was centrifuged at 4000x g for 20 min to separate the cells. The cells were resuspended in basal salts solution without an energy source. These cells were used as inoculum for ferrous ion oxidation and sulfur leaching from coal.

Accurate cell counts were achieved for experimental runs from protein analyses calibrated with visual cell counts under a phase contrast microscope equipped with a Petroff-Hausser counting chamber. The total protein was assayed by the modified Bradford Method suggested by Peterson[10].

Medium with ferrous sulfate as the energy source was prepared by adding 9 g/l or 3.18 g/l of $FeSO_4 \cdot 7 H_2O$ to the basal salts medium to yield an initial iron concentration of 2000 or 700 ppm. Experiments were conducted with and without yeast extract supplement. An uninoculated control flask was run in parallel to show the rate of chemical oxidation of ferrous iron by oxygen from the air. Samples were taken every 12-24 hours for the assay of ferrous and ferric iron concentrations. The analysis followed a procedure using 1,10 phenanthroline hydrochloride as the indicator for iron concentration. Details of the analytical procedures are available in the literature[11].

Other experiments were conducted with 5% coal slurry comprised of Kentucky #9 coal and basal salts solution. The slurry was either inoculated or uninoculated with the cells. Every 12-24 hours, 3 ml of slurry were taken from each flask and filtered through Whatman #1 filter paper. Concentrations of sulfate, ferrous, and ferric iron in the solution and total sulfur content in the coal were measured. Concentration of sulfate was determined by the turbidimetric method specified in ASTM[12]. The sulfur content in coal was measured by combustion and titration using a LECO sulfur analyzer.

KINETIC ELEMENTS

The mechanism for pyritic sulfur oxidation was first proposed by Silverman[13] for Thiobacillus species as comprised of direct and indirect oxidative components. The direct mechanism requires direct contact between bacteria and pyrite since no extracellular enzymes are involved. In the indirect mechanism, the ferric iron chemically reacts with pyrite to give ferrous iron and elemental sulfur. The bacteria then oxidize ferrous iron to ferric iron and oxidize elemental sulfur to sulfate. In the absence of bacteria, the regeneration of ferric iron is the rate limiting step for pyrite oxidation. Bacteria increase the pyrite oxidation rate by oxidizing ferrous iron to ferric iron. Precipitation of iron-sulphate was observed when ferric iron was present at high concentration.

To model the system, kinetics of the following reactions were considered:
1. Chemical oxidation of ferrous ions to ferric ions by dissolved oxygen without bacteria
2. Biological oxidation of ferrous ions by suspended microorganisms with bacteria
3. Chemical oxidation of pyrite to iron and sulfate
4. Biological oxidation of pyrite by attached microbes
5. Precipitation of sulfate and ferric ions

Microbial oxidation of pyrite by Thiobacillus ferrooxidans has been studied and Monod type models were proposed[8]. The basic concept is that the adsorbed cells grow on pyrite surfaces and the suspended cells grow on ferrous ions. An equilibrium exists between the free cells and the adsorbed cells according to the Langmuir isotherm. However, Kargi and Robinson pointed out that in their study of pyrite leaching with S. acidocaldarius, that the number of free cells remained unchanged while the attached cells grew in number[9]. Recent reports showed that in the short term, the cells attached to coal particles following the Langmuir isotherm and that selective adsorption on pyrite surfaces was evident, but irreversiblity was developed through long term cell-coal contact[14,15].

The attached cells are assumed to have a maximum specific growth rate of $\mu_{M,S6}$ which is independent of the amount of pyrite. As suggested by Huber et al.[16], the Monod type of model was not appropriate here since pyrite is not a soluble substrate. The value, X_A is the density of attached cells, defined as cells/mM pyrite, and Y_c is the yield factor for microbial oxidation of pyrite defined as cells/mM FeS_2 oxidized. The microbial growth is considered as occurring in two ways. One is the growth on pyrite surface, the other term is the growth on ferrous iron in solution. For the growth on pyrite surface, the number of attached cells is limited by a saturation cell density designated as X_A. For the growth on ferrous iron, the Monod expression can be applied. The density of the cells which did not attach to the pyrite surface (could be free suspended or attached to surfaces other than pyrite) is designated as X_F. Based on the assumptions, the rate equation for each kinetic element is proposed as follow:

Rate of chemical oxidation of Fe^{2+} = $k_1 [Fe^{2+}] - k_{-1}[Fe^{3+}]$ ....(1)

Rate of microbial oxidation of Fe^{2+} = $\dfrac{\mu_{M,Fe} * [Fe^{2+}]}{K_{Fe} + [Fe^{2+}]} * \dfrac{X_F}{Y_{Fe}}$ ....(2)

Rate of chemical oxidation of FeS_2 = $k_s[Fe^{3+}](\dfrac{[Fe^{2+}]_{eq}}{[FeS_2]_{eq}} - \dfrac{[Fe^{2+}]}{[FeS_2]})$..(3)

Rate of biological oxidation of FeS_2 = $\dfrac{\mu_{M,S} * X_A*[FeS_2]}{Y_S}$ ....(4)

Rate of Fe^{3+} precipitation = $k_p [Fe^{3+}]$ ....(5)

Based on the rate equations proposed above, balance equations for Fe(II), Fe(III), attached cells, free-floating cells and pyritic sulfur can be writen as:

$$-\frac{d[Fe^{2+}]}{dt} = -k_1([Fe^{2+}] - K_e[Fe^{3+}]) + \frac{\mu_{M,Fe}*[Fe^{2+}]}{K_{Fe} + [Fe^{2+}]}$$

$$+ 3\,k_s[Fe^{3+}](\frac{[Fe^{2+}]_{eg}}{[FeS_2]_{eq}} - \frac{[Fe^{2+}]}{[FeS_2]}) + 3\,\frac{\mu_{M,S}\,X_A[FeS_2]}{Y_S} \quad \ldots\ldots\ldots(6)$$

$$-\frac{d[Fe^{3+}]}{dt} = k_1([Fe^{2+}] - K_e[Fe^{3+}]) + \frac{\mu_{M,Fe}*[Fe^{2+}]}{K_{Fe} + [Fe^{2+}]}\frac{X_F}{Y_{Fe}} - k_p[Fe^{3+}] \ldots\ldots(7)$$

$$\frac{d\,X_A}{dt} = L_{M,S}\,X_A \quad \text{when } X_A < X_A^* \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(8)$$

$$= 0 \quad \text{when } X_A = X_A^*$$

$$\frac{d\,X_F}{dt} = \frac{\mu_{M,Fe}[Fe^{2+}]}{K_{Fe} + [Fe^{2+}]} + X_A*(-\frac{d[FeS_2]}{dt}) \quad \ldots\ldots\ldots\ldots\ldots\ldots(9)$$

$$-\frac{d[FeS_2]}{dt} = k_s[Fe^{3+}](\frac{[Fe^{2+}]_{eg}}{[FeS_2]_{eq}} - \frac{[Fe^{2+}]}{[FeS_2]}) + \frac{\mu_{M,S}*X_A*[FeS_2]}{Y_S} \quad \ldots(10)$$

$$= 2\,\frac{d[SO_4^=]}{d\,t}$$

The factor 3 came from the assumption that 3 moles of ferrous iron were
released when one mole of FeS_2 was oxidized with 2 moles of ferr   iron.

RESULTS AND DISCUSSIONS
Oxidation of ferrous ions in aqueous solution:
    In the absence of microorganism and pyrite, the equations to describe
the pure chemical oxidation of ferrous ions can be derived simply by
dropping the biological terms and sulfur turms in the balance equations for
a general bioleahing system. Equilibrium was reached in a few days and no
more oxudation occurred. A drop of total iron concentration signified
precipitation of ferric ions.

Microbial oxidation of ferrous ions
    Figure 1 demonstrates the data from a run with inoculated ferrous
sulfate medium. In the presence of microbes, the balance equation can be
derived by dropping the sulfur terms in the general equations. The results
of simulation are given in Figure 1. The experimental data indicate that
ferrous ions were completed oxidized to ferric ions with the help of
microorganisms. Ferric ion concentrations in the medium increased rapidly
at first and then dropped due to precipitation of ferric-sulfate complex.
The drop of ferrous ions was associated with growth of the microorganism.

Oxidation of pyrite in aqueous solution
    Experimental results   with uninoculated 5% coal slurry are shown in
Figure 2. An equilibrium was detected beyond which no more oxidation
occurred. Model prediction and data are shown in Figure 2. The model fits
the data to a satisfactory degree.

Microbial oxidation of pyrite

The data from batch leaching of -270+325 mesh Kentucky #9 coal was fitted with this model. As Case I, it is assumed that cells preferentially adsorbed on the pyrite surface. In other words, all the attached cells were on pyrite sites in the coal. Case II has only part of the attached cells on the pyrite surface while others were on coal surfaces.

Figure 3 shows the simulation for case I. The predicted ferrous iron concentration was higher than the experimental data. Figure 4 shows the simulation results with the assumption of case II. The predicted ferrous iron concentration in the first two days of leaching was lower than the experimental data, which means that fewer cells were utilizing ferrous iron as energy. In Figure 4 the number of cells which were attached to pyrite surface was assumed to be 10% of the the cells attached to coal particles. The model predicted the general trend, or the dynamics of the leaching system, but more information is required to develop further the model for a better fit to the experimental data.

CONCLUSION

Kinetic elements were proposed to describe the bacterial leaching system. The cells attached to pyrite sites grew on pyrite and yielded ferrous iron and sulfate as the products. The cells which were not on the pyrite sites, either attached to the coal surface or freely suspended utilized ferrous iron as an energy source. Chemical oxidation of pyrite by ferric iron and chemical oxidation of ferrous iron were also considered. The model gave a good description of the dynamics of the leaching system.

There are limitations in applying this model since assumptions were made to simplify the rate equations. Also, cells counts in the coal slurry were not accurately followed due to interference from the materials leached from coal. However, the model predicts the trends of concentration profiles of Fe(II), Fe(III) and sulfate ions in solution. More experiments with accurate cell counts is necessary to make the model more generally applicable.

Nomenclature

k_s: Rate constant for chemical oxidation of pyrite, (mM/day ppm^2)
k_1 : Rate constant for chemical oxidation of ferrous iron, (1/day)
k_{-1}: Rate constant for reduction of ferric ions, (1/day)
$\mu_{M,F}$: Maximum specific growth rate of cells on ferrous iron, 1/day)
K_{Fe}: Saturation constant in the Monod model, (mg Fe/l)
Y_{Fe}: Yield factor for cell growth on ferrous iron, (cells/nM Fe)
$\mu_{M,S}$: Specific growth rate on pyrite (1/day)
Y_S : Yield factor for growth on pyrite, (cells/mM FeS$_2$)
X_F : Free-floating cell density, (cells/ml solution)
X_A : Attached cell density, (cells/gm coal)

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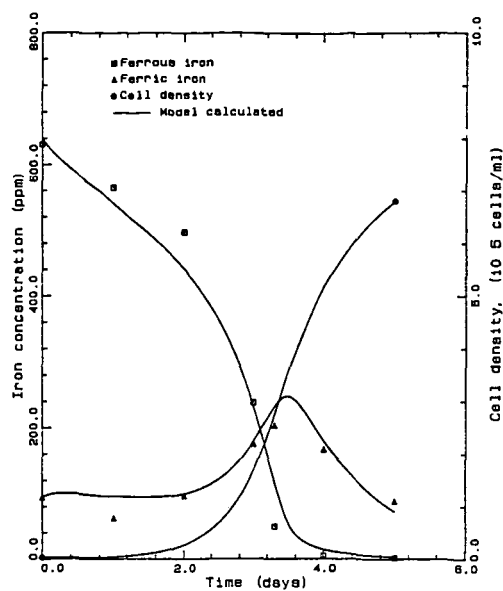
Figure 1   Curve-fitting of experimental data on microbial oxidation of ferrous iron, run #4
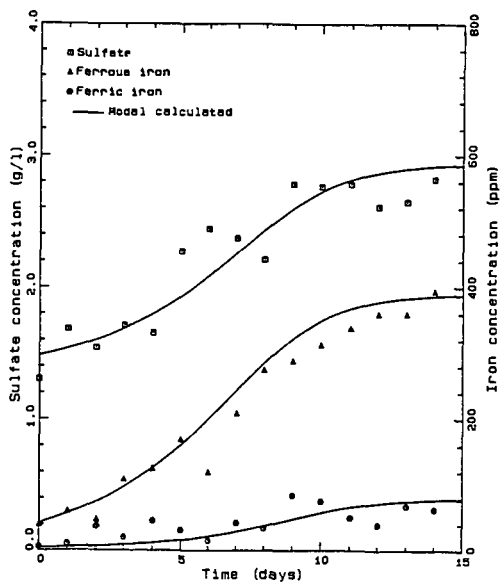

Figure 2   Curve-fitting of experimental data on an uninoculated control of 10% slurry of Kentucky #9 coal with a single dose of basal salts.
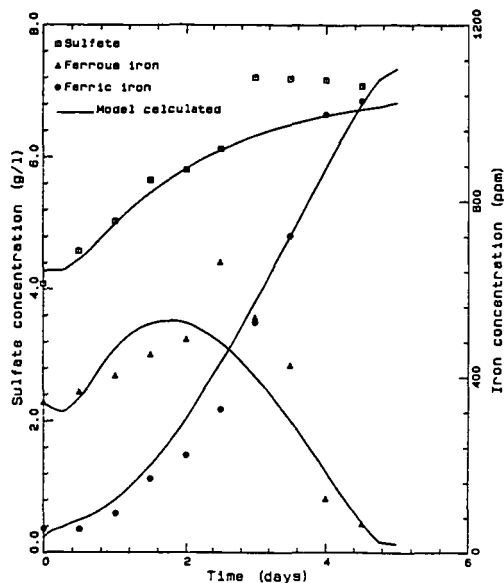
645

Figure 3. Curve-fitting of experimental data on batch
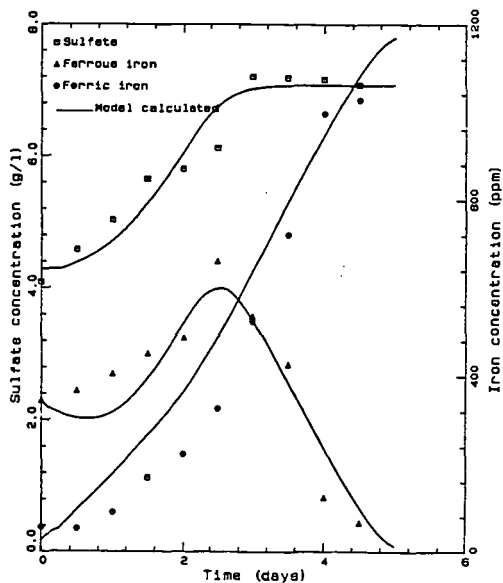leaching of -270 +325 mesh Kentucky #9 coal, case I.

Figure 4. Curve-fitting of experimental data on batch
leaching of -270 +325 mesh Kentucky #9 coal, case II.

Theoretical Problems in Modelling of Enzyme Sequences
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In vivo enzymes operate in enzyme systems[1] realizing reactions of metabolic networks. Using mechanisms of vectorial flow of matter and energy[2,3] as well as compartment and microcompartment of cells interior[4] the systems achieved high perfection[5] of catalytic action. Non-linear non-equilibrium thermodynamics describe the formation of coherent structures[6] of which enzyme systems are important example. Many of dynamic properties of the systems may be explained by control theory[7] or by more and more complicated kinetic models[8]. Measurable properties of the system reflect both properties of individual enzymes and the system as a whole and its environment. The system simultaneously realizes transport, binding and catalysis functions which are impossible to single out in the models. But rather than to bind or transport a substrate, the most important property of an enzyme is to catalyze the conversion of a substrate into a product[9]. On the other hand, the models in principle base on the approximation of an absolute continuous medium commonly used in most chemical models. This is reasonable for a large chemical system i.e. containing very large numbers of molecules but becomes doubtful for enzymes in conditions extant in vivo. What is even more important is that catalysis itself, viz. quantum mechanical interactions between a catalytic center c, a substrate s and a product p molecules is associated with the particular attributes of the objects c, s, p rather than with properties of an enzyme macrosystem and its environment.

New general models of enzyme systems, composed of elementary catalytic systems were suggested[10] for these reasons. A model of the elementary catalytic system (ECS), derived from the notions of the general theory od systems, which is capable of performing a single elementary catalytic act (ECA) is as simple as possible functional model of a system with the catalysis function only. The term „catalytic system" was first used by Rudenko[11] in his analysis of coupled catalysis and transport functions. To modelling of the optimum cooperation between many ECS´s and its environment the general models of multicatalytic system (MCS) were used[10,12]. The MCS´s were shown to contain finite numbers n of ECS. An effectiveness of an ECS action inside the MCS, described by a maximum classical probability (MPr(ECA)) of succesive ECA occurrence, increases non-linearly with increasing of n incomparison with a system of n ECS non-organized into MCS. For analysis of the catalysis function of enzyme systems the MCS models look to be very useful. It is just the purpose of the present paper to show that optimum value of n for simple MCS is about 15. In term of ECS and MCS a discussion of some modern biophysics and biotechnology problems will be made.

Elementary Catalytic System

The ECS is defined as a discrete, determinated system with purposeful behaviour which is a function of attributes of the system objects and bi-object relations. The purpose of the ECS action is to realize a sequence of bi-object events in a well-defined order (ECA). A set of the events does not contain those connected with flow of matter into and away of the ECS, which are function of environment attributes.

The ECS is composed (Figure 1) of a set of five objects which are represented by vertices of a structural graph and of the set of bi-object relations represented by the graph branches. The formal objects q and k where q is the source and k is the receiver of energy quanta simplify the modelling of energy flow inside ECS during ECA. Any object of ECS may exist in one of the states belonging to the object set of states, which is the subset of finite

minimal set of states required for ECA to occur. There are two attributes of a state x_i of object $\underline{x}$: I_{x_i} and E_{x_i}, where I_{x_i} is a constant time interval between the moments of appearance of two succesive active states (impulses) required for the reaction to occur, so called oscillation time in impulse-oscillation parametric model (IOM) of molecule[12], and E_{x_i} is the internal energy of object $\underline{x}$ in x_i state.

In ECS discrete time scale is used. All time parameters are assumed to be natural number counts of arbitrary time units. A bi--object relation (x_i, y_i) arises then and only then if a moment $SYN_{x_i y_j}$ of synchronization can be attained:

$$SYN_{x_i y_j} = a_{x_i} + n_{x_i} \cdot I_{x_i} = a_{y_j} + n_{y_j} \cdot I_{y_j} \qquad 1)$$

where: n_{x_i}, n_{y_j} are natural numbers; a_{x_i}, a_{y_j} are the moments of appearance of first active states in the system. The change of states x_i, y_i of objects $\underline{x}$, $\underline{y}$ is the result of the relation.

To describe a flow of internal energy from $\underline{x}$ object (energy donor) to $\underline{y}$ object (energy acceptor) the following model was adopted:

$$E_{x_i} + E_{y_j} = E_{x_{(i+1)}} + E_{y_{(j+1)}} \qquad 2)$$

$$E_{x_i} > E_{y_j} \qquad 3)$$

$$E_{x_{(i+1)}} \geqslant E_{y_{(j+1)}} \qquad 4)$$

where: 2) follows from the energy concervation law; 3) and 4) define energy gradients before and after the flow, respectively.

The analysis of the general ECS model showed that an ECA process is the sequence of bi-object events (Figure 2) which occur if the conditions 1)-4) are fulfilled. Results obtained with computing of detailed particular ECS model (in preparation) showed, among others, that the catalysis effect (ECA duration time smaller that the time necessary for the relation ($\underline{s}$ $\underline{p}$) to occur) is possible to achieve for particular I_{c_0} values only and that the effect increases when the difference between I_{s_0} and I_{p_0} decreases.

Models of cooperation between ECS and its environment

To make many succesive ECA´s occur, it is necessary to carry s_0 and g_0 into, and p_0 and k_2 away from the ECS. Thus, the ECS model may be simplified and considered (Figure 3) a transformer of the chemical $\underline{s}$ and the energy $\underline{g}$ input signals into the respective signals $\underline{p}$ and $\underline{k}$.

The probability of the succesive ECA occurrence is a function of those events that depend on the interaction of the appropriate inputs/outputs to the ECS with its environment because the probabilities of all bi-object events inside the ECS are equal to unity (the determined system). For the estimation of MPr(ECA) the following simplifying assumptions are made:
- the number of objects in the ECS environment and the number of inputs/outputs with which ECS and its environment cooperate are determined
- the probability P(w) of any object appearance at any ECS input/output is the same
- the objects appear at appropriate moments $\underline{a}$ (eq. 1))
- individual events are independent
- any object $\underline{s}$, $\underline{p}$, $\underline{g}$, $\underline{k}$, $\underline{c}$ may exist in one of its equally probable states (Fig. 2).

1. An ECS inside stochastic environment

   The objects number = 4; the inputs/outputs number = 4
   $MPr(ECA)_1 \approx 2 \cdot 10^{-9}$

2. $\underline{n}$ ECS inside stochastic environment

   The objects number = 4n; the inputs/outputs number = 4n
   $MPr(ECA)_2 \approx 2 \cdot 10^{-9} \cdot n^{-8}$

3. Two ECS energy-coupled inside stochastic environment
   First ECS serves as a source of energy quanta for the second. It is possible if:

   $$^1I_{k_2} = {}^2I_{g_O}; \qquad {}^1_m a_{k_2} = {}^2_m a_{g_O} \qquad\qquad 5)$$

   $$^2I_{k_O} = {}^1I_{g_2}; \qquad {}_{m+1}^1 a_{k_O} = {}^2_m a_{g_2}$$
   $$^2E_{g_O} = {}^1E_{k_2}; \qquad {}^2E_{g_2} = {}^1E_{k_O} \qquad\qquad 6)$$

   where the left superscripts and subscripts are ECS numerals in the system and ECA numerals in the ECS, respectively.
   From the energy flow model 2)-4) and 6) it follows[12] that $^1E_{C_O} > {}^2E_{C_O}$. If the number of objects and inputs/outputs are 6 then $MPr(ECA)_3 \approx 2\cdot10^{-8}$, in comparison with $MPr(ECA)_2 \approx 10^{-11}$ for $\underline{n} = 2$.

4. Two ECS chemically coupled inside stochastic environment
   The $\underline{p}$ object in p_O state from first ECS serves as the $\underline{s}$ object in s_O state inside the second ECS. It is possible if:

   $$^1I_{p_O} = {}^2I_{s_O}; \qquad {}^1_m a_{p_O} = {}^2_m a_{s_O} \qquad\qquad 7)$$

   $$^1I_{p_-} = {}^2I_{s_-}; \qquad {}_{m+1}^1 a_{p_-} = {}^2_m a_{s_-}$$

   $$^1E_{p_O} = {}^2E_{s_O} \qquad\qquad 8)$$

   $$^1E_{p_-} = {}^2E_{s_-} \qquad\qquad 9)$$

   States p_- and s_- serve to convey information of the lack of object $\overline{p}$ and $\overline{s}$ in the system. For the number of objects and inputs/outputs equal 6 $MPr(ECA)_4 \approx 2.5\cdot10^{-8}$, thus the same order as for model 3.

5. Chain MCS: $\underline{n}$ ECS energy-coupled inside stochastic environment.
   5a. The objects number $\underline{4n}$; the inputs/outputs number $(2n+2)$
       $MPr(ECA)_{5a} \approx 3\cdot10^{-4}\ [n(2n+2)]^{-2}$
   5b. The objects number $(2n+2)$; the inputs/outputs number $(2n+2)$
       $MPr(ECA)_{5b} \approx 5\cdot10^{-3}\ (2n+2)^{-4}$

   The above formulas are true for $n\geqslant3$, with terminal ECS disregarded. The absolute values of $MPr(ECA)_5$ decrease with increasing $\underline{n}$; $MPr(ECA)_{5a} \approx MPr(ECA)_1$ for $n=12$; $MPr(ECA)_{5b} \approx MPr(ECA)_1$ for $n=18$. The relative effectiveness $(MPr(ECA)_5/MPr(ECA)_2)$ increases with increasing $\underline{n}$, approximately with $\underline{n}^4$.

6. Chain MCS: $\underline{n}$ ECS chemically coupled inside stochastic environment.
   6a. The objects number $4n$; the inputs/outputs number $(2n+2)$
       $MPr(ECA)_{6a} \approx 4\cdot10^{-4}\ [n(2n+2)]^{-2}$
   6b. The objects number $(2n+2)$; the inputs/outputs number $(2n+2)$
       $MPr(ECA)_{6b} \approx 7\cdot10^{-3}\ (2n+2)^{-4}$

   The above formulas are true for $n\geqslant3$, with terminal ECS disregarded. $MPr(ECA)_{6a} \approx MPr(ECA)_1$ for $n=14$; $MPr(ECA)_{6b} \approx MPr(ECA)_1$ for $n=20$.

7. Ring MCS: $\underline{n}$ ECS chemically coupled inside stochastic environment.
   7a. The objects number $\underline{4n}$; the inputs/outputs number $\underline{2n}$
       $MPr(ECA)_{7a} \approx 1\cdot10^{-4}\ n^{-4}$
   7b. The objects number $\underline{2n}$; the inputs/outputs number $\underline{2n}$
       $MPr(ECA)_{7b} \approx 4\cdot10^{-4}\cdot n^{-4}$
       $MPr(ECA)_{7a} \approx MPr(ECA)_1$ for $\underline{n} = 15$;
       $MPr(ECA)_{7b} \approx MPr(ECA)_1$ for $\underline{n} = 21$.

Discussion

The general character of the present considerations has prompted us to make the assumptions some of which need not to be obligatorily satisfied in individual cases. The MPr(ECA) estimates should be viewed as the orders of magnitude rather than exact values. On the other hand, the formal general analysis of ECS and MCS models leads to a number of conclusions which are well consistent with the modern biophysics and catalysis. At the same time it seems that the models have a good predictive capability helpful in studies of many biotechnology problems. From this point of view, for example, one should postulate a new look to be given to carriers employed in immobilization of enzymes in order to pick up carriers capable of an additional function of directional energy flow which could significantly enhance enzyme effectiveness; the relations 5)-6) or 7)-9) may be used in experiments with immobilization of bienzyme systems (the estimated effect due to chemical coupling is by at least one order of magnitude higher than established in experimental studies reported in literature); it seems that energetical relations[12] may help in studies of the formation of new series of chemical transformations in artificial non-biological MCS systems. However to achieve this, more detailed studies in the field are required.

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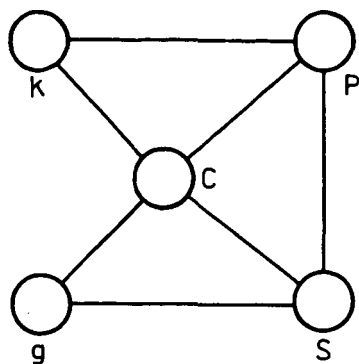
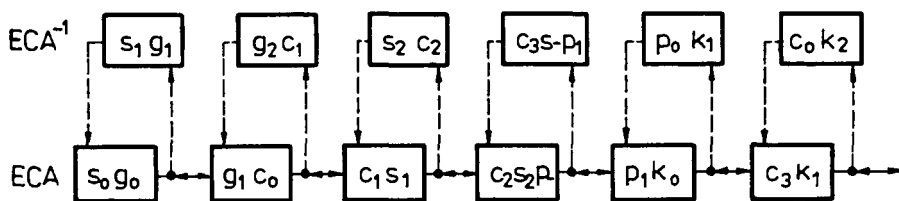Figure 1. A graph of the ECS structure.

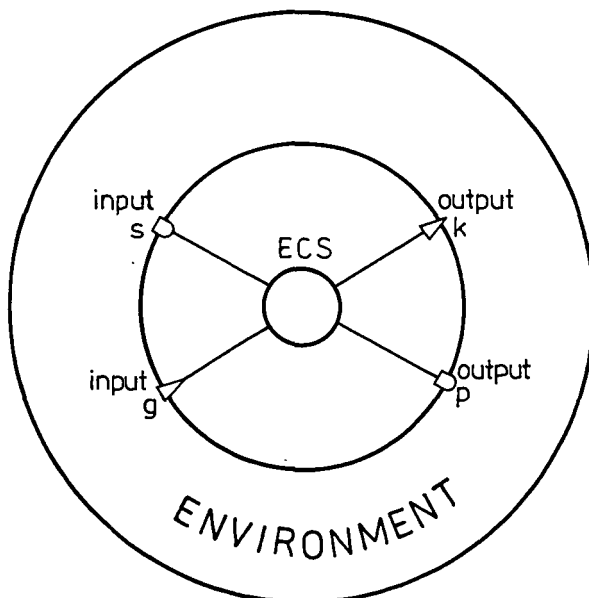Figure 2.   A diagrammatic presentation of an ECA.

Figure 3.   A simplified model of interaction between ECS and

surroundings.   651

Lignin Peroxidase-Catalyzed Depolymerization
of Water Soluble Polymer
Derived from Subbituminous Coal and Lignite

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In view of reports that enzymes can depolymerize or otherwise alter lignin, it is to be anticipated that enzymes will also attack some of the linkages in coal. However, the solid and vitreous nature of coal precludes a direct test of this possibility. For this reason, it has been necessary to prepare a coal substrate with minimal chemical alteration which is water soluble in the pH range where enzymes are active.

This report describes the preparation and characterization of a coal liquid substrate suitable for enzymes and the ability of lignin peroxidase to alter this material.

Preparation of Coal Polymer

Use was made of the well-known ability of nitric acid to oxidize low ranked coals and lignite to yield an alkali soluble material. Ground North Dakota lignite or German subbituminous coal was treated with 20% to 40% nitric acid at less than 70°C for 2 to 6 hours. The washed and dried material was suspended in 1N NaOH at room temperature for 30 minutes and then centrifuged. HCl was added to the brown-black supernatant solution to pH 3.5, and the copious brown-black precipitate, which salted out with the 1N NaCl produced on acidification, recovered by centrifugation. In an alternative procedure, the nitric acid-treated subbituminous coal was suspended in water and the pH raised to 10 to 11 with NaOH. After acidification to pH 3.5 with HCl and centrifugation, NaCl was added to the brown-black supernatant to 1M concentration and the precipitate recovered by centrifugation.

The salted out precipitate obtained in either procedure was extracted with water at room temperature for 20 minutes to 12 hours to obtain a dark-brown soluble material which was dialyzed and retained in 12,000-14,000 dalton cut off membranes.

The yield of soluble organic carbon by an acid dichromate colorimetric method was 35 to 61% based on the organic carbon content of the nitric acid-treated material.

The water soluble material was also soluble in dimethyl formamide, dimethyl sulfoxide, tetrahydrofuran, sparingly soluble in methanol, and insoluble in acetonitrile, ethyl acetate, hexane and benzene.

Gel filtration chromatography of the soluble material from North Dakota lignite on Sephadex G-75 and G-200 with effluent monitoring at 254 nm revealed a single relatively narrow peak migrating with Blue Dextran on G-75 and slightly slower than Blue Dextran on G-200. Similar results were obtained with the soluble material from German subbituminous coal by high performance gel filtration chromatography with the exception that in addition to the major peak of about 125,000 daltons, there were minor components of about 50,000 and 32,000 daltons. No low molecular weight components were present in either preparation. In dialysis experiments at 0, 1, and 3M KCl and at acidic and basic pH values, virtually all of the material was retained. From these results it is concluded that the bulk of the pH 3.5 water soluble material had a molecular weight of greater than 100,000 daltons, and as such is a satisfactory substrate for enzyme studies.

Lignin Peroxidase

Lignin peroxidase of _Phanerochaete chrysosporium_ is unique among enzymes: (a) in its ability to catalyze reactions without regard for stereoconfiguration of the substrate; and (b) in the seemingly large number of different reactions it catalyzes. In this regard both lignin and coal are appropriate substrates being stereoirregular polymers with a multiplicity of intercomponent linkages.

The partially purified lignin peroxidase used in these experiments was recovered from _Phanerochaete chrysosporium_ cultures by adsorption to and specific elution from DEAE-Sephadex coated porous silica (Macrosorb Kax.DEAE, Sterling Organics US, New York, NY) and concentration on an American YM-10 membrane (American Corporation, Danvers, MA). Manganese peroxidase activity was virtually absent from these preparations. Except for this recovery procedure, growth of the organisms, enzyme assay, etc., followed published procedures.

Incubation of Coal and Lignite Preparations with Lignin Peroxidase

Soluble coal and lignite preparations were incubated at 37°C with lignin peroxidase in tartrate buffer, pH 3.0, and H_2O_2 for 4 to 24 hours. The incubations were oxygenated periodically. Aliquots of reaction mixtures were either directly applied to conventional gel permeation columns of Sephadex G-200 or to high performance gel permeation chromatographic columns. In some analyses samples were first treated with acetone-dimethyl formamide (1:1). Following removal of denatured protein by centrifugation, the supernatant solution was recovered, dried, and dissolved in buffer for gel permeation chromatography (0.02 M KPO_4, pH 7 - 0.5% Tween 80).

The elution diagrams for soluble material from North Dakota lignite obtained by monitoring the effluent at 254 nm showed a substantial loss of the major peak for the polymer and formation of a mixture lower molecular weight components in low yield;no production of "monomeric components" was not observed. With more peroxidase and incubation at pH 4.5, the accumulation of lower molecular fragments was enhanced.

Similar results were obtained with soluble polymer from German subbituminous coal. There was a substantial disappearance of the major peak and an appearance of material of both

higher and lower molecular weight. The addition of veratryl
alcohol caused the nearly complete disappearance of the major
peak without the appearance of fragments absorbing at 254 nm.
This indicates extensive alteration of aromatic rings in the
starting material had occurred.

Discussion

The results clearly show that lignin peroxidase can
modify soluble coal polymers derived form North Dakota lignite
and German subbituminous coal, probably by several reactions
involving depolymerization and polymerization. This process
probably should be considered as distinct from the liquification
of powdered low ranked coals and lignite on mats of growing
fungi as has been reported in numerous publications. The nature
of the chemical modifications are unknown. Based on the current
concepts of the mechanism of lignin peroxidase, it is to be
expected that a donation of electrons from oxidizable groups to
the Fe(IV)-oxo form of the peroxidase will form a cation radi-
cal. This election transfer may occur either directly or via
the veratryl alcohol cation radical. Non-enzymatic rearrange-
ment of the cation radical could follow many paths depending on
the substructure involved and these could occur with or without
the introduction of oxygen. It is, therefore, to be expected
that a large number of products of both higher and lower molecu-
lar weight will be formed. Hence, it will be necessary in
future research to separate the products and to determine their
structure so as to establish the nature of the lignin peroxidase
attack on soluble coal polymer.

CHARACTERIZATION OF BIODEGRADED COALS

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INTRODUCTION

Microbial degradation of coals to materials that are soluble in water has been a topic of intensive research for the last few years. The potential for economical recovery of low-grade coals, coupled with possibilities for further upgrading by microbial desulfurization or methanation has spurred intensive research at a number of laboratories. Until very recently, coal biodegradation has been accomplished using low-grade, naturally oxidized coals such as leonardite (1,2,3), or coals subjected to pretreatment with oxidizing chemicals (4). We have been able to accomplish the biodegradation of bituminous Illinois #6 coal after a pretreatment consisting of air oxidation, using a culture of the fungus *Penicillium* sp. We report in this paper results of chemical and spectrometric analyses of the starting materials and products from Illinois #6 coal biodegradation, and compare the results with those previously reported (2) from the biodegradation of leonardite.

EXPERIMENTAL

Coal and Coal Pretreatment · Illinois #6 coal (mine-washed) was obtained from the Illinois Department of Natural Resources. Prior to microbial treatment, the coal was sized to 0.5 to 5 mm diameter, spread to a layer approximately 1 cm thick on Al foil, and heated in a forced draft oven at 150°C for 7 days. Thermocouple measurements made on the coal during pretreatment showed that the coal temperature did not deviate significantly from the oven temperature.

Coal Biodegradation -Agar plates containing Sabourauds maltose medium (Difco Laboratories, Detroit, MI) were inoculated with a strain of *Penicillium* obtained from Professor Bailey Ward of Louisiana State University. After 7 days incubation at room temperature, 0.3 to 0.4 g of pretreated Illinois #6 coal was placed on each plate, and incubation continued. Water-solubilized coal was harvested by pipette after 5 weeks, passed through 0.45 μ filters, and freeze-dried. The residual material was extracted with 50 mL portions of 0.5 $\underline{N}$ NaOH until extracts were colorless (approximately 700 mL was used per plate). The base solubilized coal was filtered (0.45 μ), acid precipitated (HCl, pH 2), and dried.

Solubilization of leonardite coal by *Coriolus versicolor* has been
described elsewhere (2).

Analysis for conversion to Soluble Products - Conversion of coal to
water or base solubles was determined by measurement of optical
density at 680 nm after appropriate sample dilution. Standards were
prepared from dry acid-precipitated subsamples of solubilized coal
product.

Elemental Analysis - Samples were dried for 48 hr under vacuum at
125°C prior to analysis. Elemental analyses were carried out by
Schwarzkopf Microanalytical Laboratory, Woodside, NY.

Gel Permeation Chromatography(GPC) - GPC was conducted in
tetrahydrofuran solvent containing 0.1% acetic acid flowing at 1
mL/min through two μStyragel columns (100 and 500 Å, Waters
Associates (Milford, MA) in series. Detection was by ultraviolet
absorbance (Waters Model 490) and by mass detector (Applied
Chromatography Systems Ltd, (Bedfordshire, England). Calibration was
with polystyrene standards, and with pyrene.

^{13}C NMR Spectroscopy - A Varian VXR-300 equipped with an ultra high
speed probe (Doty Scientific, Inc.,Columbia, SC), spinning at 14 kHz,
was used to obtain NMR spectra of coal and coal bioproducts. At the
high spin rate, no sidebands interfere within the entire spectral
window of interest. The instrument was run in the cross-polarization
mode, with a 56 kHz decoupling field.

RESULTS

Recoveries of water and base soluble material from the plates after
degradation for 5 weeks are shown in Table 1. In contrast to the
biosolubilization of leonardite, which is rendered water soluble by
C. versicolor, yields of water solubles from *Penicillium*-biodegraded
pretreated Illinois #6 coal were very low. The total yield of solid
material that could be directly pipetted from the plates was on the
order of 3%. When this material was recovered from solution by acid
precipitation rather than freeze-drying, less than half could be
recovered, an indication that the water-solubles were contaminated by
the media in which the organism was grown. Recovery of coal material
by base extraction was much more satisfactory. Although recovery
with 0.5 N NaOH is indicated to be 80 to 90% in Table 1, recent data
has shown that after 6 weeks of incubation, over 95% can be extracted
from the plates with base. The high degree of solubility in even
0.01 N base is an indication that the solubilization by base results
from a metathetical exchange of sodium ions for active hydrogens,
rather than solvolysis reactions.

The data in Table 2 indicates that the biodegraded material is
different from materials derived from base solubilization of the

air-oxidized coal. Base solubilization of air-oxidized Illinois #6 coal is only accomplished to a significant extent from 24-hr treatment with 2.4 $\underline{N}$ base. Conditions producing high yields of solubilized material from the biotreated coal give only 6% solubles from the undegraded pretreated coal. In addition, the biodegraded material has significantly different molecular weight properties than the undegraded, base solubilized oxidized coal (Table 2). Although the molecular weight (MW) ranges are similar, the base soluble biodegraded coal exhibits a weight-average MW only one-sixth that of the base solubilized oxidized coal. After recovery by acid precipitation, the water-soluble biodegraded Illinois #6 coal is essentially indistinguishable in MW properties from the corresponding base-soluble material. For comparison, the molecular weight characteristics of biodegraded leonardite are included; there is a significant difference in both weight average MW and MW range between the Illinois #6 product and the leonardite product.

Ash and elemental analyses of the Illinois #6 coal, before and after pretreatment, and the coal-biodegraded products are presented in Table 3. It is clear from the data that the pretreatment caused profound changes in the coal composition. Weight loss upon heating in air was 10%; essentially the same as when the coal is vacuum dried at 125°C. However, Table 3 shows that there was a substantial loss in both carbon and hydrogen after heating, offset by a large increase in oxygen content. The elemental compositions of the biodegraded materials were lower in sulfur, presumably because of pyrite losses during the solubilization and filtration processes. Nitrogen was somewhat elevated, probably due to some contamination with protein material. Oxygen was appreciably elevated in the water soluble product, and oxygen/carbon ratios were elevated in the products over the starting pretreated coal. Low material balances in the acid-precipitated products resulted from relatively high quantities of chlorine were present (2.6% in the water soluble product, and 7.4% in the base soluble product). Chlorine (as chloride ion) would displace metallic oxide oxygen or hydroxyl during the acid precipitation process.

^{13}C NMR spectra of untreated Illinois #6 coal, 150°C air-treated Illinois #6 coal, and the base soluble biodegradation product are shown in Figure 1. Chemical shifts obtained from carbon in different chemical environments have been well-documented in coal samples (5). The chemical shift region from 0 to 75 ppm includes resonance from carbon in aliphatic linkages; the region from 90 to 155 ppm contains resonance from carbon present in aromatic rings; from 155 to 215 ppm is found signal from carbon present in carbonyl structures. Specifically, chemical shifts in the region 165-185 ppm are assigned to carbon in carboxyl groups (6). From Figure 1, it can be seen that upon air treatment at 150°C, the aliphatic region is reduced in intensity relative to the aromatic region, and the aromatic region somewhat broadened. Upon biodegradation, the aliphatic peak is even

further reduced, while a distinct peak in the carboxyl region is produced. For the three Illinois #6 samples, quantitative determinations of the relative abundances of different carbon obtained by integration of the three chemical shift areas are given in Table 4, together with data obtained from leonardite and biodegraded leonardite.

DISCUSSION

Recent studies of the air oxidation of coal between ambient and 150°C (7, 8, 9) indicate that the incorporated oxygen is largely present in ether-type linkages rather than in carboxyl groups as might be expected. Infrared studies of coal during oxidation at temperatures between 25 and 100°C (7,8) have indicated initial formation of carboxyl groups, followed by their disappearance (presumably through thermal decarboxylation), and the evolution of ether bonds. A study of the oxidative weathering of freshly mined Illinois #6 coal (9) in which an additional 26% oxygen was incorporated over 2 months under ambient conditions, found that no carbonyl groups were present. The NMR spectrum obtained from our pretreated coal sample indicates that carboxyl groups are not in high concentration. The broadening of the peak containing chemical shifts from aromatic carbon toward higher chemical shifts in the pretreated sample (Figure 1), may be an indication of carbon involved in ether bonds, since the C-O chemical shift is in the region 148-158 ppm (5). We thus have evidence through the literature and through the NMR spectra that oxygen incorporation into Illinois coal during pretreatment is largely through ether formation, and that losses of carbon and hydrogen are through CO and CO_2 evolution, as well as losses of other volatiles.

Shown in Table 5 are the empirical formulas, based on 100 carbon atoms, obtained from elemental analyses of Illinois #6 and leonardite coals and coal products. In terms of elemental composition, the effect of the microbial action on the oxidized coal does not appear to be great. For the Illinois #6 coal 4 oxygens, 11 hydrogens, and 1 nitrogen were added per 100 carbon atoms; for the leonardite case, 2 oxygens, 7 hydrogens, and 2 nitrogens were added per 100 carbons. Although stoichiometry is not precise, the addition of the elements of water during the microbial degradation indicates that hydrolysis is involved in the biodegradation of oxidized coal. Further, an oxidative hydrolysis mechanism is suggested by the reduction in molecular weight of the microbial product, the appearance of carbonyl in the ^{13}C NMR spectra after biodegradation, the facile solubility of the product in weak base, and the finding in our laboratories that soluble enzymes produced by the coal-degrading organisms readily hydrolyze benzyl ethers and oxidize aromatic hydrocarbons (J. A. Campbell et al., presented at this symposium).

The oxidation of lignin materials has been reported to occur through mechanisms involving cleavage of beta-aryl ethers (10,11), and

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aromatic ring cleavage (12). In addition, parallel pathways of side chain degradation, decarboxylation, and aromatic ring opening have been described for the degradation of lignin model compounds (13). From our ^{13}C NMR data (Table 4), leonardite loses 10% aromatic carbon while gaining 10% C=O carbon, which is consistent with a mechanism of aromatic oxidative hydrolysis; however, there does not appear to be a loss of aryl carbon in the case of the Illinois #6 coal (Table 4). For Illinois #6 coal, ether cleavage and side chain degradation seems to be favored, since loss of aliphatic carbon is observed.

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ACKNOWLEDGMENT

Table 1. Yields of *Penicillium*-Degraded Illinois #6 Coal

Extracting Solvent	% of Biodegraded Illinois #6 Coal Extracted
Water	3.0 ±0.8 (s.d.,n=6)
0.50 N NaOH	79.0 ±10.9 (range,n=2)
0.05 N NaOH	89.4 ±2.2 (range,n=2)
0.01 N NaOH	51.0 ±6.5 (range,n=2)

Table 2. Yields and GPC-Determined Molecular Weights of Soluble Coal Fractions

Sample	Solvent Treatment	% Soluble	Wt. Av. Mole Weight	MW Range (THF Sol*)
Biodeg. Ill #6 (Insol. H₂O, sol. base)	0.05-0.5 N	100	900	200 - 100,000
H₂O-Sol Ill#6 (Acid pptd)	H₂O	100	1000	200 - 100,000
Air Ox. Ill #6 (150°C, 7 days)	2.4 N NaOH	25	6,000	200 - 100,000
Air Ox. Ill #6	0.5 N NaOH	6	-	-
Biodegraded Leonardite	H₂O	100	2,000	200 - 20,000

* Base soluble fractions were 70 to 90% soluble in the GPC solvent

Table 3   Elemental Analysis of Illinois #6 Coal
          Biodegradation Products: Comparison with Starting
          Materials and Leonardite Products

Sample	% C	% H	% O	% N	% S	% Ash	Total
ILL #6 Coal	67.43	4.08	11.8	1.5	4.35	14.16	103.32
Air Ox Coal	58.53	2.13	23.42	1.16	4.36	14.42	104.02
Base Sol Prod	52.85	2.41	23.74	1.77	2.57	10.15	93.49
H2O Sol Prod (acid precip)	56.12	2.14	29.75	3.04	2.59	2.03	95.67
Leonardite	54.95	3.6	30.24	0.8	1.06	8.32	98.97
Leon Prod (acid precip)	53.53	3.83	30.7	2.07	1.06	3.48	94.67

Table 4. Carbon-Type Analysis of Coal and Biodegraded Coal
         Samples by ^{13}C NMR

	% C=O	% Aromatic C	% Aliphatic C
Illinois #6	5	55	40
Oxidized Illinois #6	17	58	25
Biodegraded Ill #6	28	60	12
Leonardite	10	45	45
Biodegraded Leonardite	20	35	45

Table 5. Empirical Formulas Calculated for Illinois #6 Coal,
          Leonardite, and Their Biodegradation Products

Illinois #6 Coal $C_{100} H_{73} O_{13} N_{1.9} S_{2.4}$

Heat-Treated Illinois #6 Coal $C_{100} H_{44} O_{30} N_{1.7} S_{2.8}$

Base Soluble, Acid Precipitate $C_{100} H_{55} O_{34} N_{2.9} S_{1.8}$

Leonardite $C_{100} H_{79} O_{41} N_{1.2} S_{0.7}$

Water Soluble, Acid Precip. $C_{100} H_{86} O_{43} N_{3.3} S_{0.7}$
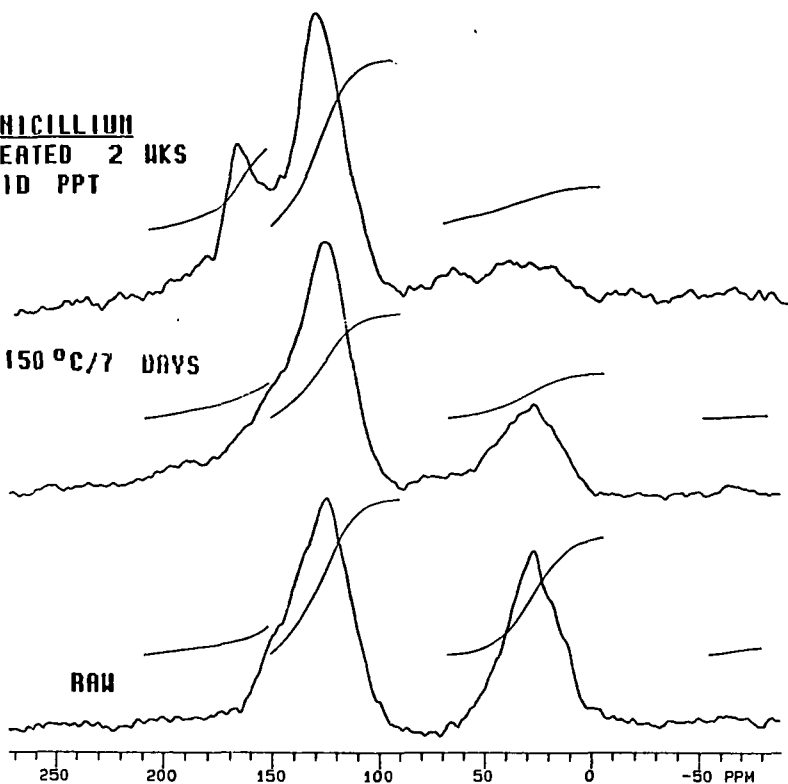
Figure 1. ^{13}C NMR spectrum of Illinois #6 coal and coal products. Bottom, untreated Illinois #6 coal; Middle, coal pretreated by heating at 150° C in air; Top, pretreated coal after degradation by the fungus *Penicillium* sp.

BIOLOGICAL REMOVAL OF HYDROGEN SULFIDE
FROM COAL SYNTHESIS GAS

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INTRODUCTION

Coal synthesis gas may typically contain up to 1% hydrogen sulfide
(H_2S) which, in conventional processing, must be removed prior to
methanation to prevent catalyst fouling (1,2). Alternatively,
Barik et al (3) have shown that coal synthesis gas may be upgraded
microbially in a process which is not particularly sensitive to
H_2S. However, H_2S would have to be removed from the resulting
upgraded gas prior to distribution and sale.

The work described here was initiated to investigate the
feasibility of a microbial process for the removal and oxidation of
H_2S from a gas stream such as the product of the shift conversion
of coal gas or the product of the microbial methanation process.
The high reaction rates and mild reaction conditions characteristic
of a microbial process can potentially yield technical and economic
advantages over conventional processes. Conceptually, a microbial
process can replace an entire conventional gas processing train of
H_2S removal (amine system), H_2S disposal (Claus or Stretford
system) and tail gas clean-up or any individual processing step.

There are many bacteria capable of H_2S oxidation and therefore
serve as potential candidates to form the basis of a microbial gas
desulfurization technology. However, the ideal microorganism for
this application must possess several other characteristics in
addition to the ability to oxidize hydrogen sulfide if the
technology is to be economically viable. The bacteria chosen for
this study was Thiobacillus denitrificans.

T. denitrificans is a strict autotroph and facultative anaerobe
first described by Baalsrud and Baalsrud (4). Thiosulfate,
elemental sulfur and soluble sulfide may be utilized as energy
sources with oxidation to sulfate which accumulates external to the
cells. Under anaerobic conditions nitrate may be used as a
terminal electron acceptor with reduction to elemental nitrogen.
Reported here is a study of the stoichiometry and kinetics of
aerobic and anaerobic oxidation of $H_2S(g)$ by T. denitrificans in
batch and continuous flow reactors under sulfide-limiting
conditions. The maximum loading of the biomass for aerobic and
anaerobic oxidation of H_2S was determined and a study of reactor
upset conducted. The effect of heterotrophic contamination was
also examined. Lastly, T. denitrificans has also been immobilized
by co-culture with floc-forming heterotrophs to produce macroscopic
floc with excellent settling properties. A study of the oxidation
of H_2S by immobilized T. denitrificans is also reported.

MATERIALS AND METHODS

Organism and Stock Cultures

Stock cultures of wild-type (ATCC 23642) and sulfide-tolerant
strains of Thiobacillus denitrificans were grown anaerobically in
thiosulfate maintenance medium in 10 ml culture tubes at 30°C.
Thiosulfate medium is described in Table 1. The trace metal
solution has been described elsewhere (5). Stocks were transferred
every 30 days and stored at 4°C until used.

Oxidation of H_2S by Planktonic or Free-Cell T. denitrificans

All investigations of anaerobic growth of planktonic T.
denitrificans on $H_2S(g)$ as the energy source were conducted with a
B. Braun Biostat M bench scale fermenter (culture volume, 1.4 l).
Aerobic experiments also made use of an L. E. Marubishi MD 300
fermenter (culture volume, 2.0 l). In a typical anaerobic batch
experiment, T. denitrificans was grown in thiosulfate maintenance
medium at 30°C and pH 7.0 to an OD_{460} of 0.5-0.8 prior to the
introduction of H_2S. This optical density (OD) corresponds to
greater than 10^8-10^9 cells/ml. The purpose of this prior
cultivation on thiosulfate was to develop a sufficient
concentration of biomass in the reactor so that an appreciable rate
of $H_2S(g)$ could be fed to the reactor without exceeding the
biooxidation capabilities of the biomass. Otherwise toxic levels
of sulfide would accumulate in the culture. The OD level of 0.5-
0.8 was initially chosen arbitrarily. While growing on thiosulfate
a gas feed of 5 mol% CO_2 in nitrogen was normally fed to the
reactor at 30 ml/min to ensure continuous availability of a carbon
source. A hydrogen sulfide feed gas typically contained 0.5-1.0
mol% H_2S, 5 mol% CO_2, and balance N_2.

The pathways for sulfide and thiosulfate oxidation to sulfate in T.
denitrificans are not independent but have two common inter-
mediates, a membrane bound polysulfide and sulfite (6). In the
presence of thiosulfate, the rate of sulfide oxidation would be
reduced because of competition between intermediates of thiosulfate
and sulfide oxidation for the same enzymes of the sulfur pathway.
Therefore, prior to the introduction of H_2S to T. denitrificans
cultures, residual thiosulfate was removed. This was accomplished
by sedimenting the cells by centrifugation, washing with 20 mM
phosphate buffer (pH 7.0) and resuspending the cells in the
fermenter in thiosulfate maintenance medium without thiosulfate.
The cell suspension was then purged for 1 h with 5 mol% CO_2 in
nitrogen to allow residual thiosulfate to be metabolized before the
feed gas was changed to include hydrogen sulfide. H_2S oxidizing
cultures were also maintained at 30°C and pH 7.0.

The start-up of a continuous-flow, anaerobic reactor began with the
establishment of a batch culture as described above. When stable
operation on $H_2S(g)$ feed was indicated, the reactor was switched to
a continuous flow mode by introducing a continuous stream of
nutrient solution (thiosulfate maintenance medium without
thiosulfate) and withdrawing a continuous stream of reactor mixed
liquor (cells plus medium) at the same rate. A schematic diagram

of the continuous flow reactor system used in these experiments is given in Figure 1.

In batch, aerobic experiments, T. denitrificans was grown in thiosulfate maintenance medium without nitrate at 30°C and pH 7.0 until the thiosulfate was depleted. Aeration was provided at a rate sufficient to maintain 60-100 μM O_2 in the culture medium. Air was supplemented with 5 mol% CO_2 to ensure a continuous availability of a carbon source. When the thiosulfate was depleted the optical density (at 460 nM) of the culture was approximately 1.0 and the T. denitrificans viable count was approximately 10^9 cells/ml. Following thiosulfate depletion the culture received two gas feeds, 0.9-1.1 mol% H_2S, 5 mol% CO_2 and balance nitrogen and air supplemented with 5 mol% CO_2. The aeration rate and agitation rate (300-400 rpm) were adjusted to produce steady-state oxygen concentration of 60-150 μM in the culture medium. The aeration rate was typically in the range of 200-400 ml/min. Foaming was controlled with the addition of 0.5 ml of a 1/10 dilution of General Electric AF93 silicone antifoam emulsion (previously autoclaved at 121°C, 205 kPa) approximately every 18-24 h. All other details of the operation of aerobic batch reactors were identical to those characteristics of the operation of anaerobic, batch reactors described earlier.

With the exception of providing aeration, the start-up and operation of continuous-flow aerobic reactors was virtually identical to the anaerobic systems. The nutrient feed solution was identical to the thiosulfate maintenance medium described in Table 1 with the exceptions that there was no thiosulfate or nitrate in the feed.

Oxidation of H_2S by Immobilized T. denitrificans

T. denitrificans was immobilized by co-culture with floc-forming heterotrophs obtained from activated sludge from the aerobic reactor of a refinery wastewater treatment system. T. denitrificans cells grown aerobically on thiosulfate and washed sludge were resuspended together in fresh thiosulfate maintenance medium without nitrate. The culture was maintained in a fed batch mode at pH 7.0 and 30°C with a gas feed of 5% CO_2 in air. With respect to the growth of T. denitrificans this medium was thiosulfate-limiting. When thiosulfate was depleted, the agitation and aeration were terminated and the flocculated biomass allowed to settle under gravity. The supernatant liquid was then removed and discarded. In this way the culture was enriched for T. denitrificans cells which had become physically associated with the floc. The volume was then made up with fresh medium and aeration and agitation restarted. This fed-batch cycle was repeated 5-6 times.

At the end of the fed batch cycles the biomass was allowed to gravity settle and was washed four times with 20 mM phosphate buffer (pH 7.0) to remove free-cell biomass. Floc were then resuspended in fresh medium (without thiosulfate) in the fermenter and sparged with 500 ml/min of 5% CO_2 in air and 50 ml/min of 1.0% H_2S in nitrogen.

<u>Analytical</u>

All gas analyses were conducted with a Perkin Elmer Sigma I Gas
Chromatograph or Hewlett-Packard 5995 GC/MS. The Perkin Elmer
featured a thermal conductivity detector with a detection limit for
H_2S of 2-4 μM with a 0.25 ml sample at 101.3 kPa and 25°C. The
column used was a 10-ft by 1/8-in. ID Teflon column containing
80/100 mesh Porapak QS (Waters Associates). The detection limit of
the HP GC/MS for H_2S was found to be approximately 0.05 μM with a
0.25 ml sample at 101.3 kPa and 25°C. The column used was a 6-ft
by 1/8-in. ID glass column containing 60/80 mesh Tenax GC (Alltech
Associates).

Whole cell protein was determined by sonication followed by protein
determination by the micro-modification of the Folin-Ciocalteau
method (7,8). The protein content of <u>T. denitrificans</u> cells was
determined to be 60% $\pm$ 3% by dry weight. Using this figure,
protein analyses were converted to dry weight <u>T. denitrificans</u>
biomass.

Nitrate was determined by the cadmium reduction method and nitrite
by the diazotization method (9). Ammonium ion was determined by
the Nessler method without distillation (9). Thiosulfate was
determined by titration with standard I_2 solution with a starch
indicator (10). Sulfate was determined turbidiometrically (9).

Total sulfide (H_2S, HS^- and S^{-2}) was determined by ion specific
electrode using an Orion Research Model 94-16 sulfide/silver
electrode and an Orion Research Model 701A pH/mV meter. Elemental
sulfur collected by filtration on 0.45-μ Millipore Type HA filters
was determined by reaction with cyanide to produce thiocyanate
which was quantitated as $Fe(SCN)_6^{-3}$ (11).

Viable counts of <u>T. denitrificans</u> were determined by plating serial
dilutions of culture medium on thiosulfate agar plates and
incubating anaerobically at 30°C. Heterotrophs were quantitated by
plating samples of culture medium on nutrient agar (Difco Labs)
plates incubated aerobically at 30°C.

Free-cell or planktonic biomass in reactors containing immobilized
<u>T. denitrificans</u> was estimated in terms of the optical density (460
nm) of the supernatant after medium samples were allowed to settle
under gravity for 10 min. Settling properties of flocculated
biomass were determined during the course of H_2S fermentation by
periodically turning off all gas feeds and the agitation and
measuring the height of the biomass in the fermenter at intervals
for 20 min.

RESULTS AND DISCUSSION

<u>Batch Growth of Planktonic T. denitrificans on H_2S</u>

When H_2S was introduced to anaerobic or aerobic cultures of
planktonic or free-cell <u>T. denitrificans</u> previously grown on
thiosulfate, the H_2S was immediately metabolized with no apparent

lag. At initial loadings of 4-5 mmoles/h-g biomass the reactor
outlet gas generally contained less than 2.0 μM H_2S. When H_2S was
detected in the outlet gas, an increase in the agitation rate
reduced the H_2S concentration to levels undetectable by GC/MS. The
residence time of a bubble of feed gas (average diameter
approximately 0.25 cm) under these conditions was 1-2 s. Less than
1 μM of total sulfide (H_2S, HS^-, S^{-2}) was observed in the reactor
medium during periods of up to 36 h of operation. No elemental
sulfur was detected; however, sulfate accumulated in the reactor
medium as H_2S was removed from the feed gas (Figure 2). Oxidation
of H_2S to sulfate was accompanied by growth, as indicated by an
increase in optical density and protein concentration and a
decrease in the NH_4^+ concentration as shown in Figures 2 and 3.
Consumption of OH^- equivalents indicated that the reaction was acid
producing. Nitrate was consumed under anaerobic conditions as
expected (Figure 3), but no nitrite was observed to accumulate.

Sample material balances for batch aerobic and anaerobic H_2S
oxidation are given in Table 2. Table 3 presents this type of data
as stoichiometric ratios all relative to H_2S oxidized. The
stoichiometries of aerobic and anaerobic oxidation of H_2S(g) by T.
denitrificans in batch reactors were similar with respect to
sulfate production and ammonium and hydroxide utilization.
However, the biomass yield under aerobic conditions was only about
37% of that observed under anaerobic conditions. These results
suggest that oxygen may be a growth inhibiting substrate for T.
denitrificans growing on H_2S(g). Similar results have been
reported by Justin and Kelley (12) for aerobic growth of T.
denitrificans on thiosulfate.

Growth of Planktonic T. denitrificans on H_2S in a CSTR

T. denitrificans was readily cultured both anaerobically and
aerobically on a continuous basis with a H_2S feed. Less than 2
mg/l of elemental sulfur was deterected in the reactors at any
time. Nitrite was detected in some anaerobic reactors during
start-up (up to 0.25 mM in one instance); however, no nitrite could
be detected in any anaerobic reactor at steady state. The steady
state concentration of total sulfide in each reactor was less than
or equal to 1 μM. This was true throughout each experiment even
during start-up. Typical behavior in approach to steady state in
an anaerobic CSTR is illustrated by Figures 4 and 5. Small amounts
of N_2O (< 40 μM) resulting from the incomplete reduction of nitrate
were detected in the outlet gases from anaerobic reactors. With
sufficient agitation H_2S was undetectable by GC/MS in the outlet
gases of both aerobic and anaerobic reactors.

The stoichiometry of anaerobic and aerobic H_2S oxidation in
continuous cultures is also given in Table 3. Under anaerobic
conditions the yield of biomass was seen to be greater at the
higher dilution rate as expected since a greater fraction of energy
yielding substrate will be consumed to support growth as opposed to
cell maintenance at higher growth rates. The true growth yield and
maintenance coefficient for anaerobic H_2S oxidation by T.
denitrificans were calculated by the method given by Pirt (13) to
be 21.1 g dry wt biomass/mole H_2S and 1.74 mmoles H_2S/h-g dry wt,

respectively. At D=0.029 h^{-1} the average yield was 9.3 g
biomass/mole H_2S oxidized. This compares favorably with that
reported by Timmer-ten-Hoor (14,15) (9.41 g biomass/mole S^{-2}) for
anaerobic growth of T. denitrificans on Na_2S as limiting substrate
at D=0.030 h^{-1}. Under aerobic conditions biomass yield was
relatively unaffected by the specific growth rate in the range of
0.030 h^{-1} to 0.053 h^{-1}. As indicated in Table 3 biomass yield under
aerobic conditions in continuous cultures was lower than that
observed under anaerobic conditions at comparable dilution rates.

Reactor Upset and Recovery

In those experiments described above, the H_2S feed rate was always
less than the maximum rate at which the biomass was capable of
oxidizing the substrate. If the maximum capacity of the biomass
for H_2S oxidation is exceeded, inhibitory levels of sulfide will
accumulate in the medium. In order to examine the behavior of a T.
denitrificans reactor in an upset condition, the H_2S feed rate to
aerobic and anaerobic batch and continuous flow reactors like those
described in the previous sections was increased in a stepwise
manner until H_2S breakthrough was obtained. At the point at which
breakthrough occurred, N_2O was also detected in the outlet gas from
anaerobic reactors in concentrations approximately equal to that of
the H_2S in the feed gas. Analysis of the reactor medium from both
aerobic and anaerobic reactors also indicated an accumulation of
sulfide and elemental sulfur in the reactor. Sulfur balances for
reactors operated under upset conditions showed that all of the H_2S
removed from the feed gas could be accounted for in terms of
sulfate, elemental sulfur, and sulfide in the medium. It was
observed that the upset condition was reversible if the cultures
were not exposed to the accumulated sulfide for more than 2-3 h.
Reduction in H_2S feed rate following an upset condition reduced H_2S
and N_2O concentrations in the outlet gas to preupset levels. In
addition, elemental sulfur, which accumulated during upset, was
rapidly oxidized to sulfate.

It is of importance to know at which H_2S loadings the specific
activity of the T. denitrificans biomass will be exceeded resulting
in upset. The maximum loading of the biomass under anaerobic
conditions was observed to be in the range of 5.4-7.6 mmoles/h-g
biomass. Under aerobic conditions, the maximum loading was
observed to be much higher, 15.1-20.9 mmoles H_2S/h-g biomass.

Effect of Heterotrophic Contamination

The autotrophic medium used in these experiments will not support
the growth of heterotrophs since there is no organic carbon source.
However, early on in this study it was observed that if aseptic
conditions were not maintained, a heterotrophic contamination
developed in a T. denitrificans culture. Evidently T.
denitrificans releases organic material into the medium in the
normal course of growth or through lysis of nonviable cells which
supports the growth of heterotrophs. In order to investigate the
effect of heterotrophic contamination on the performance of a T.
denitrificans CSTR, one anaerobic reactor which became contaminated
was allowed to operate for an extended period of time. The reactor

was originally contaminated by two unidentified gram-negative
heterotrophs which had distinctly different colony morphology on
nutrient agar. After 145 h of operation, the reactor was injected
with suspensions of four facultatively anaerobic heterotrophs in
phosphate buffer. These were <u>Pseudomonas chlororaphus</u> ATCC 9446,
<u>Pseudomonas stutzeri</u> ATCC 11607, <u>Pseudomonas fluorescens</u> ATCC
33512, and an unidentified gram-negative lab isolate. After 315 h
of operation, a sample of <u>Desulfovibrio desulfuricans</u> ATCC 13541 in
phosphate buffer was also injected. The <u>Desulfovibrio</u> quickly
washed out. However, the total heterotroph concentration increased
to about 10^8 cells/ml and leveled off. Apparently growth of the
contaminants became limited by the availability of suitable carbon
sources. The viable count of <u>T. denitrificans</u> at steady state was
5.0×10^9 cells/ml. The steady state composition of the culture
medium and outlet gas condition were indistinguishable from that of
a pure culture of <u>T. denitrificans</u> operated under the same culture
conditions. These observations led to the efforts to immobilize <u>T.
denitrificans</u> by co-culture with floc-forming heterotrophs
described below.

Oxidation of H_2S by Immobilized T. denitrificans

The activated sludge used as a source of floc-forming heterotrophs
in the immobilization of <u>T. denitrificans</u> contained many
morphological forms of bacteria as well as fungi and protozoa.
However, at the end of the fed-batch cycles (18 days on the
average) in co-culture with <u>T. denitrificans</u> in thiosulfate
maintenance medium, microscopic examination revealed only short
(0.5 μ by 1.0-1.5 μ), gram-negative rods. <u>T. denitrificans</u> and
<u>Zoogloea ramigera</u> are both short gram-negative rods. <u>Zoogloea
ramigera</u> is the most common floc-forming bacterium found in
activated sludge systems.

When H_2S(g) was introduced into batch aerobic reactors containing
immobilized <u>T. denitrificans</u>, the H_2S was immediately metabolized.
With gas-liquid contact time of approximately 1-2 sec the outlet
gas of the reactor typically contained less than 0.1 μM H_2S.
Sulfate was observed to accumulate in the medium as H_2S was removed
from the feed gas. The oxidation of H_2S was accompanied by growth
as indicated by an increase in the total protein concentration and
a decrease in the NH_4^+ concentration. In a typical experiment the
oxidation of 205.5 mmoles H_2S was accompanied by the production of
204.9 mmoles sulfate and 614.7 mg biomass protein and the utili-
zation of 16.2 mmoles NH_4^+. This stoichiometry is comparable to
that given in Table 3 for the aerobic oxidation of H_2S by
planktonic or free-cell <u>T. denitrificans</u> biomass.

As noted above, each time fermenters were sampled the sample was
allowed to settle under gravity for 10 min and the optical density
of the supernatant measured. At the time H_2S feed was initiated
the OD_{460} was typically 0.25-0.32. As H_2S was removed from the feed
gas the OD after settling was observed to actually decline
indicating that no free-cell biomass accumulated in the culture
medium. It appears that the growth of the autotroph <u>T.
denitrificans</u> was balanced with the growth of the floc-forming
heterotrophs through a commensal relationship in which the growth

of the heterotrophs was limited by organic carbon derived from $T.$ $denitrificans$. The result was an immobilization matrix which grew with the $T.\ denitrificans$.

Figure 6 gives typical settling curves for the immobilized $T.$ $denitrificans$ biomass during the course of growth on H_2S. As shown here the settling properties of the biomass were relatively constant during these experiments. When the fed-batch cycles are coupled with the H_2S experiments, the settling properties of the biomass are seen to have been maintained for in excess of 25 days without external addition of organic carbon.

CONCLUSION

It has been demonstrated that the H_2S content of a gas can be reduced to very low levels by contact with an aerobic or anaerobic culture of planktonic or free-cell $Thiobacillus\ denitrificans$ if the reactor is operated under sulfide-limiting conditions. Hydrogen sulfide was observed to be an inhibitory substrate; however, upset conditions produced by excess H_2S feed were readily detected and reversed. Hydrogen sulfide was oxidized completely to sulfate. Under aerobic conditions the maximum loading of the biomass was 2-3 times higher than that observed for anaerobic conditions.

$T.\ denitrificans$ has also been immobilized in macroscopic floc by co-culture with floc-forming heterotrophs from an activated sludge treatment facility. Floc with excellent settling characteristics were produced which were subsequently used to remove H_2S from a gas stream bubbled through a batch aerobic culture. No organic carbon addition was required during enrichment for immobilized cells of $T.$ $denitrificans$ or during H_2S oxidation. No free-cell biomass was released into the medium during growth on H_2S. The stoichiometry and kinetics of H_2S oxidation by immobilized $T.\ denitrificans$ were comparable to that observed in free-cell cultures. Immobilization of $T.\ denitrificans$ will facilitate concentration of cell suspensions by gravity settling and cell recycle in a continuous system. A corresponding increaase in volumetric productivity is anticipated.

ACKNOWLEDGEMENT

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TABLE 1. Growth Medium for <u>Thiobacillus denitrificans</u>

Component	per liter	
Na_2HPO_4	1.2	g
KH_2PO_4	1.8	g
$MgSO_4 \cdot 7H_2O$	0.4	g
NH_4Cl	0.5	g
$CaCl_2$	0.03	g
$MnSO_4$	0.02	g
$FeCl_3$	0.02	g
$NaHCO_3$	1.0	g
KNO_3 (Anaerobic)	5.0	g
$Na_2S_2O_3$ (Grow up only)	10.0	g
Trace metal solution	15.0	ml
Mineral water	50.0	ml

TABLE 2. Material Balances: Aerobic and Anaerobic Oxidation of $H_2S(g)$ in Batch Reactors by <u>T. denitrificans</u>

	Anaerobic	Aerobic
H_2S oxidized	18.3 mmoles	86.0 mmoles
SO_4^{-2} produced	18.8 mmoles	81.8 mmoles
Biomass produced	246 mg	453 mg
NO_3^- consumed	27.0 mmoles	–
NH_4^+ consumed	2.2 mmoles	8.4 mmoles
OH^- consumed	31.8 meq	151.3 meq

TABLE 3. Stoichiometry of H_2S Oxidation by <u>Thiobacillus denitrificans</u>[a]

Reactor Type	Electron Acceptor	NO_3^-/H_2S (mole/mole)	O_2/H_2S (mole/mole)	SO_4^{-2}/H_2S (mole/mole)
Batch	NO_3^-	1.36		1.04
CSTR D = 0.029 h^{-1}	NO_3^-	1.30		1.03
CSTR D = 0.058 h^{-1}	NO_3^-	1.19		1.00
Batch	O_2		1.81	0.99
CSTR D = 0.030 h^{-1}	O_2			1.06
CSTR D = 0.053 h^{-1}	O_2			1.04

TABLE (continued)

Reactor Type	Electron Acceptor	NH_4^+/H_2S (mole/mole)	OH^-/H_2S (eq/mole)	Biomass/H_2S (g/mole)
Batch	NO_3^-	0.12	1.60	12.1
CSTR D = 0.029 h^{-1}	NO_3^-	0.09	1.37	9.3
CSTR D = 0.058 h^{-1}	NO_3^-	0.10	1.24	12.9
Batch	O_2	0.10	1.75	4.5
CSTR D = 0.030 h^{-1}	O_2	0.11[b]	2.38[b]	8.1
CSTR D = 0.053 h^{-1}	O_2	0.12	1.77	7.9

[a] Average of three or more determinations unless otherwise noted.
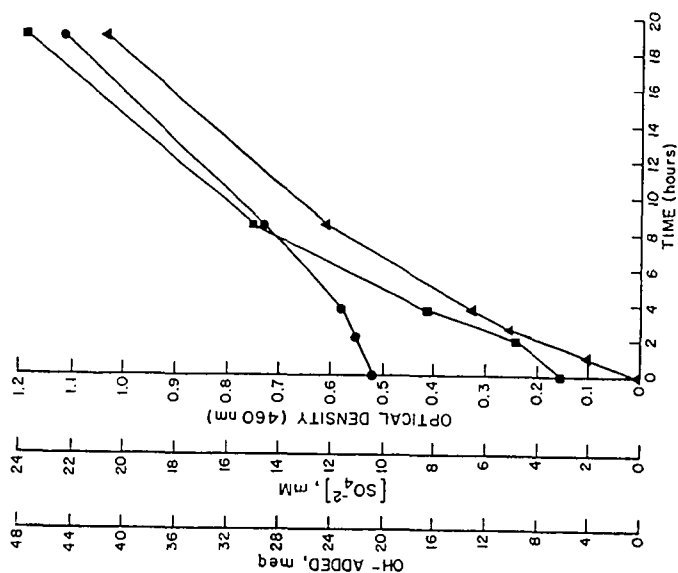[b] Average of two determinations.

FIGURE 2. Optical Density, Concentration of Sulfate (SO_4^{-2}) and Hydroxide Ion (OH^-) Utilized in an Anaerobic $T.$ $denitrificans$ Batch Reactor Receiving 1.25 $mmole/hr$ Hydrogen Sulfide (H_2S) Feed. OD $(\bullet)$; SO_4^{-2} $(\blacksquare)$; OH^- $(\blacktriangle)$.
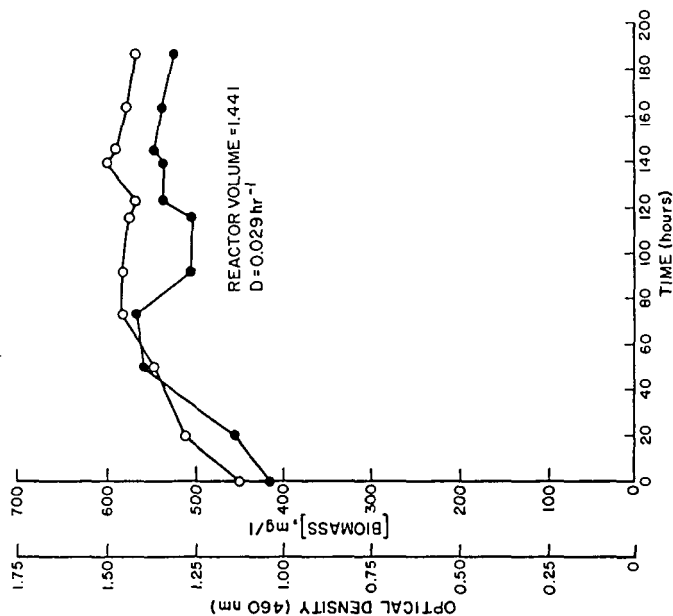

FIGURE 1. Schematic Diagram of Continuous Flow Fermentation System.

FIGURE 4. Concentration of Biomass and Optical Density in an Anaerobic T. denitrificans CSTR Receiving 2.6 mmoles/hr Hydrogen Sulfide (H$_2$S) Feed. OD (O); Biomass (●).
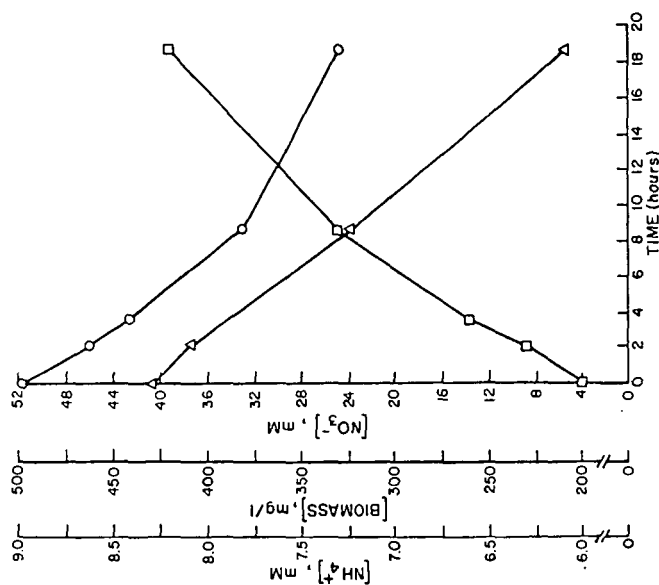
FIGURE 3. Concentrations of Nitrate (NO$_3^-$), Biomass and Ammonium (NH$_4^+$) in an Anaerobic T. denitrificans Batch Reactor Receiving 1.25 mmoles/hr Hydrogen Sulfide (H$_2$S) Feed. NO$_3^-$ (O); NH$_4^+$ (△); Biomass (□).
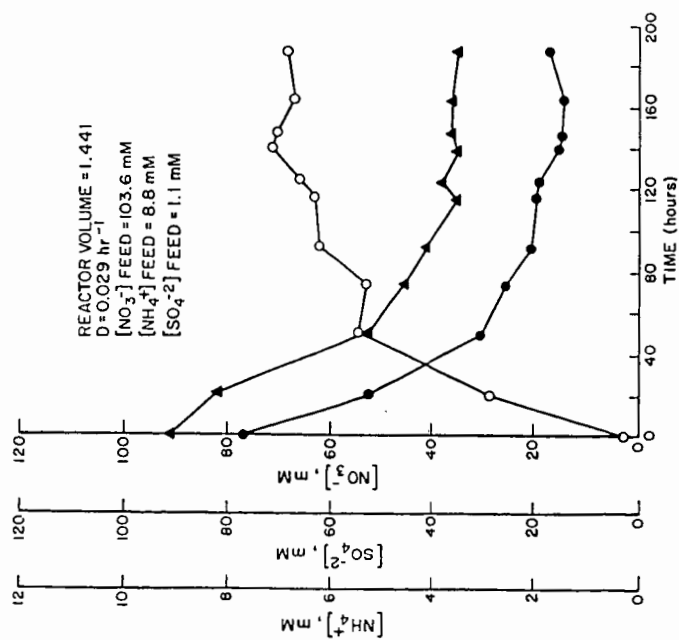
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FIGURE 6. Height of Biomass in Settling Test in Marubishi MD 300 Fermenter.

FIGURE 5. Concentrations of Nitrate (NO_3^-), Sulfate (SO_4^{-2}) and Ammonium (NH_4^+) in an Anaerobic _T. denitrificans_ CSTR Receiving 2.6 mmoles/hr Hydrogen Sulfide (H_2S) Feed. NO_3^- (●); SO_4^{-2} (○); NH_4^+ (▲).
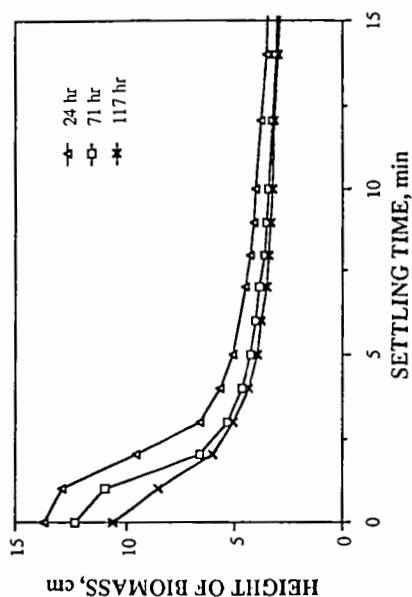
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Biological Production of Liquid and Gaseous Fuels from
Coal Synthesis Gas

by

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Abstract

Cultures of microorganisms have been isolated that convert CO, H_2 and
CO_2 in coal synthesis gas into methane or ethanol. The reactions are severely
mass transfer limited and bioreactor design will be a critical factor in the
application of this technology. This paper presents results of culture isolation
studies and development of continuous reactors for these cultures. The results
of bubble columns and stirred tank reactors are presented and discussed. Methods
for defining mass transfer coefficients and intrinsic kinetics are presented.
Operation of these gaseous fermentations at high pressure has enabled complete
conversion in reaction times of a few minutes.

INTRODUCTION

Coal synthesis gas represents an excellent raw material for the production of
chemicals and fuels. A typical composition of coal-derived synthesis gas
includes 25-35 percent hydrogen, 40-65 percent carbon monoxide, 1-20 percent
carbon dioxide, 0-7 percent methane and other compounds in small quantities such
as sulfur (as H_2S or COS), chlorine, etc. Many coal gasification processes exist
or are currently under development that can produce synthesis gas economically.

Chemical catalytic processes are known to utilize syngas to produce a large
variety of compounds such as methanol, formaldehyde, and acetic acid (Courty and
Chaumette, 1978). Microorganisms may also be used to convert synthesis gas
components into more desired products such as acetate, methane, and alcohols.
Biological processes, although generally slower than purely chemical reactions,
have several advantages over catalytic processes, such as higher specificity,
higher yields, lower energy costs and possibly higher resistance to poisoning.
Furthermore, the irreversible character of biological reactions allows complete
conversion and avoids thermodynamic equilibrium relationships.

The biological conversion of synthesis gas components to methane and liquid
fuels involves contacting the gas and microorganisms in liquid culture. The gas
must then absorb into the gas-liquid interface and diffuse through the culture
medium to the cell surface to be consumed by the microbes. For sparingly soluble
gases such as carbon monoxide, oxygen, etc. in contact with suspended cells it

has been well established that the main resistance to transport lies in the liquid film (Tsao and Lee, 1977; Blanch, 1979; Yoshida, 1982). The rate of transport from the gas phase into the culture medium is, therefore, faster for higher partial pressures in the gas phase. In the case where the overall reaction rate is transport controlled, that is, the dissolved gas concentration in the liquid phase is zero, the rate of transport, and thus the rate of reaction, is proportional to the partial pressure in the gas phase. Contacting schemes to maximize gas-liquid contact are thus very important.

The purpose of this paper is to present the results of laboratory experiments carried out in various contacting schemes for converting synthesis gas components to methane, acetate and ethanol. The batch reactor and two continuous reactors, the continuous stirred tank reactor (CSTR) and the bubble column, are employed. Also, the effects of increased pressure on improving mass transfer and microorganism performance are presented and discussed.

BIOLOGICAL SYNTHESIS GAS CONVERSION

Methane Production

The primary reactions in the biological conversion of synthesis gas to methane are the formation of methane precursors and biomethanation of the precursors. Table 1 shows the known biological routes to methane from synthesis gas components. All of these reactions are carried out anaerobically and usually require very low redox potentials in the liquid medium in which the microorganisms are suspended (Ljungdahl and Wiegel, 1986). As is seen in the table, the formation of methane can be accomplished by direct conversion of CO, CO_2 and H_2 or by the indirect formation of methane intermediates (acetate or H_2 and CO_2). Of the one-step reactions, only reaction I.3, the direct formation of methane from H_2 and CO_2, has been well-studied and verified (Escalante-Semerena et al. 1984). This reaction is known to be carried out by most of the methanogens (Jones et al., 1987), although some methanogens such as Methanothrix sp. are not capable of this conversion (Huser et al. 1982).

The one-step reactions that convert carbon monoxide directly to methane have been suggested in the literature. Methanobacterium thermoautotrophicum has been reported to produce methane from carbon monoxide according to Equation I.1 (Daniels et al. 1977). The growth of M. thermoautotrophicum on CO was reported to be very slow and was inhibited by high substrate concentrations. It has also been reported that other methanogenic bacteria may convert carbon monoxide and hydrogen directly to methane according to Equation I.2 (Fisher et al. 1932; and Stephenson and Strickland, 1933). It is more likely, however, that the carbon monoxide reduction to methane in these experiments proceeded via the multiple-step reactions II.3 and I.3 (Daniels et al. 1977; and Kluyver and Schnellen, 1947).

With the exception of Equation I.3, an indirect formation of methane seems more viable than the direct routes previously discussed. These multi-step reactions may involve the formation of a liquid intermediate, acetate, or the utilization of carbon monoxide to produce carbon dioxide and hydrogen by the water gas shift reaction (Equation II.3). In the latter case, the products hydrogen and carbon dioxide can be directly converted to methane (Equation I.3) or may enter the multiple step process that produces acetate as an intermediate

(Equation II.2). The organisms _Rhodopseudomonas gelatinosa_ (Uffen, 1976; and Dushekvicz and Uffen, 1979), and _Rhodospirillum rubrum_ (Breed _et al_. 1977) are known to perform the water gas shift reaction.
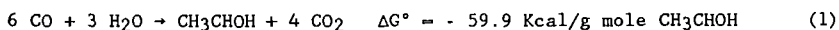
Another approach to indirect methane production is the formation of acetate as a methane precursor. In anaerobic digestion processes, 80 percent of the methane is produced from acetate by Equation II.4. The organisms _Peptostreptococcus productus_ and _Eubacterium woodii_ (Genther and Bryant, 1983) have been found to produce acetate by Equation II.1. Among these bacteria, _P. productus_ has shown the fastest growth rate and the highest tolerance to carbon monoxide. These microorganisms have also been found to carry out the conversion of hydrogen and carbon dioxide to acetate (Equation II.2), although in _P. productus_, carbon monoxide appears to be a preferred substrate.

Acetate can be transformed by methanogens of the Methanosarcinaceae family such as _Methanosarcina barkeri_ as well as _Methanothrix soehngenii_ (Jones _et al_. 1987). While _Methanosarcina barkeri_, for example, will utilize acetate only in the absence of other preferred substrates (such as H_2 and CO_2), _Methanothrix_ sp. does not utilize normal methanogenic substrates and growth and methane formation is exclusively observed in the presence of acetate (Huser _et al_. 1982). Both microorganisms show comparable specific growth rates at low acetate concentrations (< 3mM). On the other hand, in view of the Monod saturation constants available for the two microorganisms, ($K_s = 0.7$ mmol/l for _Methanothrix_), it is expected that at low acetate concentrations _Methanothrix_ is the more predominant of the two.

Ethanol Production

While many anaerobic, facultatively anaerobic and even some strictly aerobic microorganisms form various amounts of ethanol from glucose (Weigel, 1980), no organism was known to form ethanol autotrophically from synthesis gas components. In 1987, a strict anaerobic mesophilic bacterium was isolated from animal waste that was capable of converting CO, H_2, and CO_2 to a mixture of acetate and ethanol (Barik _et al_. 1987). Preliminary identification studies have indicated that the bacterium has a strong possibility of being a new clostridium species (Tanner, 1988). It is likely that in the same manner as with other clostridia growing on sugars, ethanol and acetate are formed from acetyl-CoA by this organism, with product distribution highly dependent on the regulation of electron flow (Rao, _et al_. 1987).

The overall stoichiometry for the formation of ethanol from carbon monoxide and hydrogen/carbon dioxide has been established by Vega _et al_. (1988):

$$6 \ CO + 3 \ H_2O \rightarrow CH_3CHOH + 4 \ CO_2 \qquad \Delta G^\circ = -59.9 \ Kcal/g \ mole \ CH_3CHOH \qquad (1)$$

$$2 \ CO_2 + 6 \ H_2 \rightarrow CH_3CHOH + 3 \ H_2O \qquad \Delta G^\circ = -23.2 \ Kcal/g \ mole \ CH_3CHOH \qquad (2)$$

Acetate formation from CO, CO_2 and H_2 by the organism is carried out using the same stoichiometric equations presented in Table 1.

BIOREACTOR DESIGN

Gas-Liquid Mass Transfer Concepts in Bioreactors

The transfer of gases in fermentation systems involves three phases: gas, culture medium and microbial cells suspended in the medium. In general, a combination of the following resistances can be expected (Bailey and Ollis, 1977):

1.- Diffusion from the bulk gas to the gas-liquid interface;
2.- Movement through the gas-liquid interface;
3.- Diffusion of the solute through the relatively unmixed liquid region (film) adjacent to the bubble into the well mixed bulk liquid;
4.- Transport of the solute through the bulk liquid to a second stagnant film surrounding the microbial species;
5.- Transport through the second unmixed liquid region associated with the microbes;
6.- Diffusive transport into the microbial floc, mycelia, or particle, if appropriate. (When the microbes take the form of individual cells, this resistance disappears); and,
7.- Consumption of the solute by biochemical reaction within the microorganism.

As is the case with the conventional chemical engineering analysis of absorption processes, interfacial resistance to mass transfer can be neglected. In systems where agitation is provided, transport through the bulk liquid is assumed to be instantaneous. Finally, when individual cells are suspended in a medium, the liquid film resistance around the cells is usually neglected with respect to other resistances because of the minute size and the enormous total surface of the cells (Finn, 1954). Thus for the transfer of sparingly soluble gases such as CO and H_2, the main resistance to transport lies in the liquid film. Reaction schemes should thus concentrate on minimizing the liquid film resistance of the gas transfer into the liquid phase. Other techniques for promoting gas-liquid mass transfer should also be considered, including the use of alternative liquid phases and the use of high pressure to promote higher solubility.

The conversion of carbon monoxide to acetate by reaction II.1 (see Table 1) using P. productus was chosen as a model system for bioreactor comparisons. The mass transfer limited concepts shown for this organism are applicable to other gas phase fermentation systems.

The Batch Reactor

Batch fermentation systems are typically used in determining fermentation kinetics by following the concentration of substrate in the liquid phase with time. For gas phase systems, however, the liquid phase concentration cannot be measured since sensors are not generally available for most systems. A method has been developed for determining mass transfer and intrinsic kinetic parameters in gas phase fermentation systems for sparingly soluble gases such as CO and H_2. In a typical experiment, batch reactors are started with different initial gas, partial pressures, and monitored with time for gas consumption, cell density and product formation.

Figure 1 presents the volumetric rate of disappearance of CO from the gas phase using _P. productus_ as a function of its partial pressure. The data for each partial pressure shows a period of increase in the rate of uptake of carbon monoxide from the gas phase while the partial pressure of the gas decreased slowly. During this period and because the cell concentration is low, the reaction rate is mainly under kinetic control, since the concentration of substrate in the liquid culture is above zero. The actual shape of these curves in the figure bear no physical meaning and the continuous lines drawn correspond to a best visual fitting of the data. As the cell concentration reached a value at which mass transfer controlled, the concentration of carbon monoxide in the liquid became zero and the reaction rate was that of the rate of transport of the substrate into the liquid phase. This rate of transport is then proportional to the partial pressure of carbon monoxide in the liquid phase and the proportionality constant is K_La/H. Because the fermentation conditions for all experiments (medium composition, agitation, temperature, etc.) were very similar if not the same, all the data in Figure 1 followed a single straight line. The value of the slope on the straight line as obtained from least squares of all data in the mass transfer limited region was 5.91 mmol CO/L·hr·atm. Taking for H the value for water at 37°C (H = 1.21 atm·L /mmol CO), the calculated value for K_La was 7.15 hr-1. Once the volumetric mass transfer coefficient is known, intrinsic kinetic parameters may be estimated using the data in the region where mass transfer does not control. Typically, Monod-type kinetics are used, utilizing calculated values of dissolved CO in the liquid phase.

The Stirred Tank Reactor

The traditional reactor used in fermentation processes is the continuous stirred tank reactor or CSTR. As it relates to gas phase substrates, the CSTR has continuous gas flow into a constant volume liquid phase reactor. A smaller liquid feed stream is utilized to supply nutrients to the microorganism in the reactor system. The agitation rate in the system is relatively high in order to promote transfer of the sparingly soluble gas into the liquid·culture medium.

Experiments have been conducted with _P. productus_ in a CSTR at different gas flow rates in order to develop suitable equations for modeling and process scale-up. The model which includes material balances for carbon monoxide, methane, and carbon dioxide in the gas phase and for the carbon dioxide/bicarbonate equilibrium system in the liquid medium. The model assumes that the carbon dioxide in equilibrium with the gas phase carbon dioxide partial pressure and the bicarbonate and pH level in the liquid.

Figures 2 and 3 show the solution of the model for various volumetric mass transfer coefficients (Figure 2) and various total operating pressures (Figure 3). Experimental data at 1 atm and a mass transfer coefficient of 30 are also included in the figures. As observed in the model results, increases in the mass transfer coefficient or in total operating pressures lead to higher reactor productivities. However, due to the perfect mixing in a CSTR, complete conversion is only attained when the gas flow rate is zero. The use of the model allows the extrapolation of performance of the CSTR system and permits preliminary economic evaluation of coupled with suitable equations for scale-up of properties such as the mass transfer coefficient.

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Bubble Columns

Bubble columns are commonly used in industrial processes both as reactors or absorbers whenever a large liquid retention time and/or a large liquid hold-up is needed. Some advantages of bubble columns are the lack of moving parts, minimum maintenance, relatively low costs, high interfacial area and a high mass transfer coefficient (Charpentier, 1981). The principal disadvantages are a large extent of backmixing and coalescence. These two drawbacks can be minimized by employing packing inside the column.

The performance of a bubble column for CO conversion to acetate by _P. productus_ is shown in Figure 4. The bubble column is capable of achieving similar rates of CO conversion as the stirred tank reactor without agitation. In addition, the bubble column is capable of yielding complete conversion due to plug flow operation.

By combining a CO material balance along the column with the rate expression for CO transport into the liquid phase, the following expression for the partial pressure of CO leaving the reactor is obtained:

$$\ln P_{CO}^{o} = \ln P_{CO}^{i} - \frac{K_L a}{H} \frac{\epsilon_L h RTS}{G} \tag{1}$$

where ϵ_L = fraction of liquid in the column;
  h = height of the column;
  S = cross-sectional area of the column;
  R = ideal gas constant;
  T = absolute temperature; and
  G = gas flow rate

Verification of Equation (1) is shown in Figure 5 for the data of Figure 4. As is shown, a single straight line is obtained, indicating that the model satisfactorily predicts column performance. In addition, K_{La}/H can be obtained from the slope of the line in Figure 5, once the numerical values of the constants in Equation (1) are supplied. This mathematical model can also be used for process design and scale-up in a similar manner as the CSTR model.

Pressure Effects

As was shown in Figure 3, an increase in the operating pressure brought about a significant increase in the rate of CO uptake in a CSTR. Thus, operation at increased pressures can be highly beneficial in minimizing reactor volume requirements. However, CO has been shown to inhibit growth and CO uptake at dissolved CO tensions of 0.8-1.0 atm. Methods to avoid CO inhibition by maintaining low dissolved CO tensions at increased pressures must be developed. The key to maintaining low dissolved CO tensions at increased pressures is to develop high cell concentrations that are capable of uptaking the increased quantities of CO. Higher cell concentrations can be achieved by feeding alternate substrates such as glucose, or by gradually increasing the cell concentration in a stepwise fashion.

When _P. productus_ inside a high pressure Parr reactor with an initial cell concentration of about 18 mg/L was directly pressurized to a total pressure of 5.7 atm (carbon monoxide partial pressure of 3.6 atm), no sign of carbon monoxide consumption was observed after 93 hours. On the other hand, when the pressure was raised stepwise with small increases in the carbon monoxide partial pressure at 10 hr time intervals, carbon monoxide consumption occurred at total pressures as high as 14.6 atm (carbon monoxide partial pressure of 9.3 atm). Figure 6 shows a carbon monoxide disappearance profile for _P. productus_ grown on CO where a 14.6 atm total pressure (9.3 atm carbon monoxide partial pressure) was achieved in five smaller pressure increments. As is seen, carbon monoxide was consumed at fast rates even at these high pressures. Thus, low dissolved CO tensions were achieved by gradually increasing cell growth. A proportional decrease in reactor volume is then possible through increased pressure operation.

CONCLUSIONS

Anaerobic bacteria have been utilized in the biological conversion of sparingly soluble gas phase substrates such as CO and H_2 to the products methane, ethanol and acetate. Several reactor designs may be utilized in these conversions including batch reactors and continuous stirred tank and bubble column reactors. The batch reactor has been utilized to obtain mass transfer correlations for the mass transfer controlled regime, and intrinsic kinetic parameters have been obtained in the non-mass transfer limited regime.

The continuous stirred tank reactor has been utilized to develop suitable equations for modeling and process scale-up. The model includes material balance equations and carbon dioxide/bicarbonate chemical equilibrium. The effects of the mass transfer coefficient and total pressure on CO uptake and conversion were modeled.

The bubble column has also been utilized for CO utilization. Higher conversions and similar rates of CO conversion were obtained without the required agitation in the CSTR. The results were successfully modeled and the resultant mass transfer coefficient obtained.

The benefits of increased pressure on CO utilization were demonstrated in batch and continuous culture. A gradual stepwise procedure was utilized in increasing the cell concentration to achieve low dissolved CO tensions.

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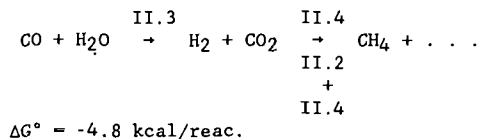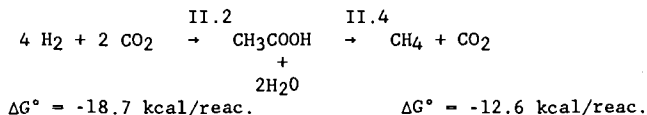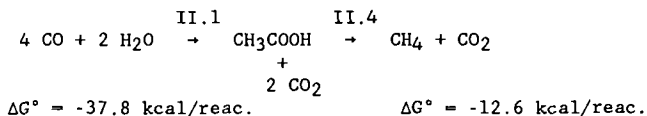
Table 1

Biological Routes to Methane From Synthesis Gas Components

I) ONE-STEP REACTIONS

I.1
$$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2 \qquad \Delta G^\circ = -54.4\ kcal/reac.$$

I.2
$$CO + 3\ H_2O \rightarrow CH_4 + H_2O \qquad \Delta G^\circ = -36.0\ kcal/reac.$$

I.3
$$4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O \qquad \Delta G^\circ = 31.3\ kcal/reac.$$

II) MULTIPLE-STEP REACTIONS

II.1   II.4
$$4\ CO + 2\ H_2O \rightarrow CH_3COOH \rightarrow CH_4 + CO_2$$
$$+$$
$$2\ CO_2$$
$$\Delta G^\circ = -37.8\ kcal/reac. \qquad \Delta G^\circ = -12.6\ kcal/reac.$$

II.2   II.4
$$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH \rightarrow CH_4 + CO_2$$
$$+$$
$$2H_2O$$
$$\Delta G^\circ = -18.7\ kcal/reac. \qquad \Delta G^\circ = -12.6\ kcal/reac.$$

II.3   II.4
$$CO + H_2O \rightarrow H_2 + CO_2 \rightarrow CH_4 + \dots$$
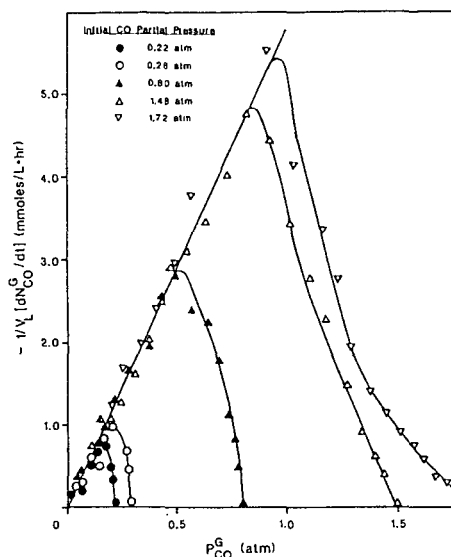II.2
$$+$$
II.4
$$\Delta G^\circ = -4.8\ kcal/reac.$$
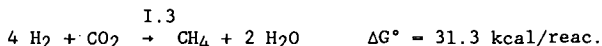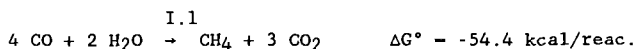
Figure 1. Determination of the volumetric mass transfer
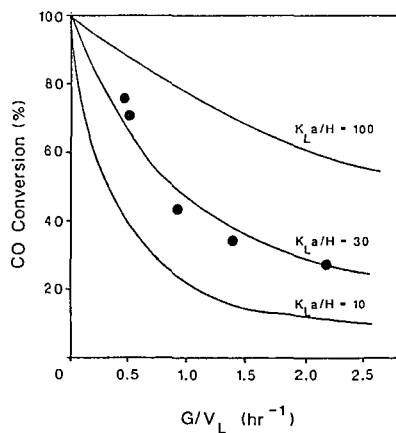coefficient for P. productus in batch culture.

Figure 2. Model results for carbon monoxide conversion
as a function of the gas flow rate per unit
of culture volume for various volumetric mass
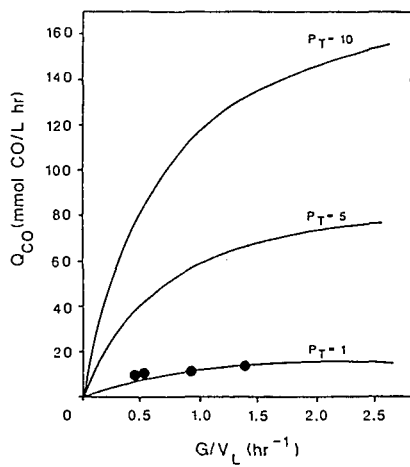transfer coefficients ($K_L a/H$ in mmol CO/atm L hr).

Figure 3. Model of results for carbon monoxide uptake as a function of the gas flow rate for various total operating pressures (P_T in atm).
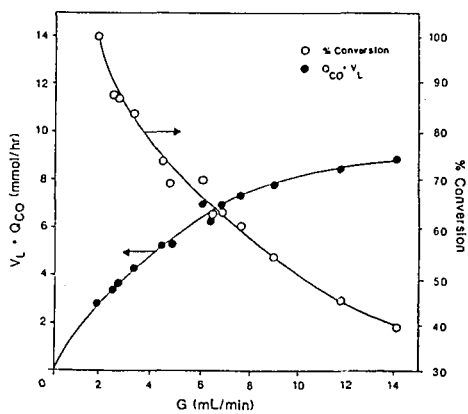

Figure 4. Carbon monoxide uptake rate and conversion level as a function of gas flow rate in the column for *P. productus*.
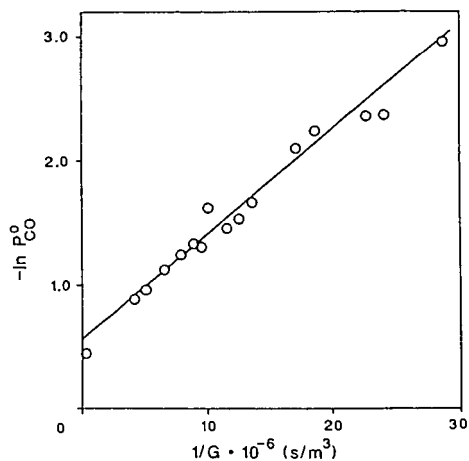
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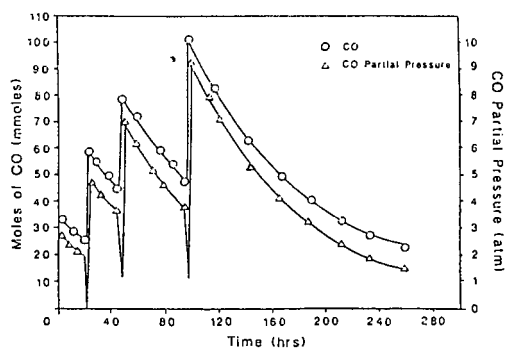Figure 5. Testing the proposed bubble column model of Equation 1.

Figure 6. CO consumption and CO partial pressure as a function of time for P. productus start-up with CO alone.